EXPRESSION OF PORCINE MESSENGER RIBONUCLEIC ACIDS ENCODING PROTEINS OF THE INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM IN MULTIPLE TISSUES DURING FETAL AND POSTNATAL DEVELOPMENT AND IN MAMMARY GLANDS DURING PREGNANCY: RELATIONSHIPS TO SERUM LEVELS OF IGFs AND IGF-BINDING PROTEINS AND ROLES FOR IGFs AND ESTROGEN IN MAMMARY DEVELOPMENT

By

CHUL-YOUNG LEE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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I dedicate this dissertation to my parents with deep appreciation for their support of my early education.

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participated in my studies with their patience and therefore should share in this achievement with me.

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CHUL-YOUNG LEE

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To elucidate the ontogeny of porcine insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) in serum and to relate this to tissue expression of the IGF system, levels of IGFs and IGFBPs in serum and of mRNAs encoding IGFs, IGFBPs, and IGF receptors and their proteins in skeletal muscle, liver, kidney, lung, and brain were measured using Northern hybridization, RIAs, affinity-crosslinking, and Western ligand blot analysis. Circulating IGF-I levels were lower than IGF-II levels by an order of magnitude during fetal life and levels of both IGFs increased postnatally. IGFBP-2 was the predominant IGF carrier in fetal serum and was replaced by IGFBP-3 postnatally. IGF-I and IGFBP-3 mRNAs were constitutively expressed during development in all tissues examined, whereas levels of IGF-II and IGFBP-2 mRNAs declined during the postnatal period. Levels of both Types I and II IGF receptors and their mRNAs

declined postnatally at all sites examined, with the exception of the lung. Results indicate that the postnatal increases in serum IGF levels are likely due to reduced clearance rather than to increased hepatic production and secretion rates of these peptides in the pig, which may be related to the postnatal transition of IGFBP-2 to IGFBP-3 in plasma and reduced expression of IGF receptors in tissues. The objective of the last experiment was to gain insights into roles for IGFs and estrogen in mammary development of gilts. Levels of mRNAs in mammary glands encoding IGFs, IGF receptors and GH receptors were higher during mammogenesis (days 12-90) than during lactogenesis (day 90-term) in pregnant (Px) gilts. Pseudopregnant (Ppx) gilts, which are known to have reduced mammary growth relative to Px gilts, had reduced levels of mRNAs encoding IGFs and IGF and GH receptors during days 60-112 which was associated with premature accumulation of ß-casein mRNA by day 60. Estradiol administration (5 mg/day) from day 45 increased ß-casein mRNA levels at days 60 and 112 in Ppx gilts. Results support roles for IGFs and estrogen in mammogenesis and lactogenesis, respectively.

CHAPTER 1

INTRODUCTION

Growth is an important phenomenon that is directly related to the efficiency of animal production. Understanding the molecular regulation of growth is therefore of great interest to animal scientists and should allow for new ways to manipulate animal growth. Growth can be defined in several ways. Hafez (1969) defined growth as the increase in linear size, weight, accumulation of adipose tissue, and retention of nitrogen and water. This definition differs from that of growth rate which refers to the per cent increase in mass and/or length during a given period. Growth rate declines, while tissue mass and length increase, as the animal grows. At the cellular level, growth can be defined as hyperplasia or hypertrophy. The hyperplastic type of growth is more important in the fetal and early postnatal periods than in later development, since retardation during the hyperplastic growth phase (fetal stage) can permanently diminish an animal's subsequent growth potential. As an example, "runt" pigs, which have reduced DNA accretion compared to normal littermates at birth, have permanent growth retardation (Widdowson, 1971).

The postnatal growth of animals is regulated by a host of hormones (Bauman et al. 1982; Etherton and Kensinger, 1984; Flint, 1987; Cabello and Wrutniak, 1989). Growth hormone (GH) plays a key role in postnatal animal growth regulation. It is well-documented that severe growth retardation occurs during GH deficiency. Conversely, excess GH secretion

causes gigantism or acromegaly (Baxter, 1986; Daughaday and Rotwein, 1989). Thyroid hormones also play an important role in growth regulation, especially in long bone growth (Glasscock et al., 1992). However, these hormones are more permissive than regulatory in nature, since hyperthyroidism is associated with an accelerated protein degradation and loss of body weight (Goldberg et al., 1980; Cabello and Wrutniak, 1989). Both estrogen and testosterone stimulate growth in domestic animals (Galbraith and Topps, 1981). These hormones increase nitrogen retention and hasten long bone growth (Lee, 1988). However, gonadal hormones may play a minor role in growth until the stage of sexual maturity. Glucocorticoid hormones, in contrast, are established growth inhibitors (Luo and Murphy, 1989).

Prenatal growth appears to be regulated primarily by genetic factors (Van Sickle, 1985; Black, 1988). Genetic factors allow fetal tissues to grow independent of other parts of the body (Van Sickle, 1985) and this may be mediated via growth factors which are expressed from earliest stages of embryonic development (Mercola and Stiles, 1988). Nutritional status also affects fetal and postnatal growth, but except for extreme situations, nutrition appears to play mainly a permissive role in growth (Chard, 1989). Fetal growth also appears to be largely independent of systemic hormonal regulation. Although placental lactogen is implicated as a fetal growth factor in the rat (Adams et al., 1983), normal delivery weights have been reported after rare human pregnancies in which placental lactogen in the circulation was totally absent (Simon et al, 1986). Further, in species, such as the pig, where placental lactogen has not been identified, fetal growth must be independent of this protein. Chard

(1989) reviewed roles for several endocrine systems involved in fetal growth and concluded that only the insulin-like growth factors are likely to be fetal growth regulators, whereas the other endocrine systems are more permissive than regulatory. In line with this view, Chard (1989) interpreted the correlation between birth weights and cord blood IGF-I concentrations in the human (Gluckman et al., 1983a) as indirect evidence supporting the regulatory role for this peptide during fetal growth.

Insulin-like growth factors (IGFs) -I and -II are structurally and functionally related to insulin and mediate the growth-promoting actions of GH (Daughaday and Rotwein, 1989). These factors are produced in multiple tissues primarily under the control of GH and act in an autocrine/paracrine as well as endocrine fashion (D'Ercole et al., 1984; Daughaday and Rotwein, 1989; Sara and Hall, 1990). Whereas most growth factors have a limited diversity of target cells, IGFs act on a variety of cell types. Partially purified IGFs have been reported to increase total body weight as well as weights of internal organs (Holder et al., 1981). IGFs are thus regulators of overall body growth. They also regulate the metabolism of three major energy substrates. These factors stimulate amino acid and glucose transport, glycolysis, and protein and fat synthesis while inhibiting degradation of protein and fat. However, not all of the actions of GH are mediated via IGFs, since GH directly suppresses fat deposition. There is also evidence for direct action of GH on skeletal tissues (Isaksson et al., 1982). This hormone preferentially stimulates the growth of muscle and skeletal tissues, resulting in increased weight gain, but a decreased net energy retention (Black, 1988).

It is generally believed that the liver produces the bulk of circulating IGFs in response to GH, while nonhepatic tissues produce primarily autocrine/paracrine-acting IGFs (Baxter, 1986; Daughaday and Rotwein, 1989; Sara and Hall, 1990). In the rat, there are several lines of evidence supporting this. Hepatic IGF-I mRNA levels and serum IGF-I concentrations increase postnatally in parallel with the increasing hepatic GH receptor binding capacity (Maes et al., 1984; Lund et al., 1986; Donovan et al., 1989). In addition, large amounts of IGF-I are secreted from cultured primary rat hepatocytes or perfused rat livers (Schwander et al., 1983; Scott et al., 1985a). However, in nonrodent species, evidence is lacking to support the concept that the liver is the major endocrine source of IGFs. An exception to this is the demonstrated correlation between hepatic GH receptor binding capacity and serum IGF-I concentrations (Gluckman et al., 1983b; Breier et al., 1989; Badinga et al., 1991).

The IGFs are found in biological fluids in association with IGF-binding proteins (IGFBPs) (Baxter and Martin, 1989a). IGFBPs potentially serve as IGF carriers, as an IGF storage site and as modulators of IGF bioactivities. As for the IGFs, serum IGFBPs are believed to be primarily of hepatic origin (Scott et al., 1985a). There are two types of IGF receptors at the cell surface. The Type I IGF receptor usually has the highest affinity for IGF-I with lower affinities for IGF-II and insulin (Massague and Czech, 1982; Rechler and Nissley, 1985). The Type II IGF receptor, which has two distinct binding sites for IGF-II and mannose-6-phosphate (MacDonald et al., 1988), has the highest affinity for IGF-II, low or no affinity for IGF-I, and no affinity for insulin (Massague and Czech, 1982; Sara and Hall, 1990).

The GH-IGF-I axis is believed to mature postnatally based upon two lines of experiments. First, fetal hypophysectomy or decapitation does not affect fetal growth in laboratory animal species (Eguchi, 1961; Chez et al., 1970). Second, hepatic GH receptor levels are very low in fetuses (Gluckman et al., 1983b). Recent reports, however, suggest that IGF expression in fetuses may be under partial control of the pituitary. Reduced serum IGF-I concentrations have been reported for decapitated porcine fetuses (Jewell et al., 1989) and for hypophysectomized fetal lambs (Mesiano et al., 1989).

The developmental expression of mRNAs encoding IGFs has been well characterized in the rat (Lund et al., 1986). In brief, hepatic IGF mRNA levels are temporally correlated with serum IGF levels (Moses et al., 1980; Donovan et al., 1989). However, virtually no comparable information is available for the pig and other large animal species. Therefore, the objectives of the present study were 1) to characterize developmental expression of IGFs, IGFBPs, IGF receptors and their mRNAs in tissues and serum, 2) to evaluate the liver as the major source of circulating IGFs and IGFBPs, and 3) to gain insights into the maturation of the somatotropic axis in the pig during fetal and postnatal development using a combination of techniques including radioimmunoassays, Northern blot hybridization, affinity-crosslinking, and Western ligand blot analysis.

IGFs have probable roles in reproduction. In the uterus, IGF-I is a possible mediator of estrogen-induced uterine growth and differentiation (Murphy and Friesen, 1988; Simmen et al., 1990) and of conceptus development during the peri-implantation period (Simmen et al., 1989b; Hofig et al., 1991a). In addition, IGFs promote the proliferation and

differentiation of the ovarian granulosa and theca cells (Adashi et al., 1989; Caubo et al., 1989).

Lactation is an integral part of mammalian reproductive function whereby the offspring is nurtured. Unlike the growth of other body tissues, mammary gland growth is greatest during pregnancy and declines with cessation of lactation as well as with senescence of reproductive functions (Forsyth, 1991). Pregnancy-associated mammary development is therefore important for the survival and development of the neonate. Although it was established many years ago that mammary development in nonpregnant rats requires gonadal and adrenal steroids as well as pituitary hormones (Lyons, 1958), roles for these hormones in pregnancy-associated mammary development in large animals are not clear. Recently, various peptide growth factors were identified in mammary glands and were therefore implicated in mammary growth and differentiation (Forsyth, 1989). In this regard, IGFs have been reported to stimulate the proliferation and lactogenesis of mammary epithelial cells in vitro (Prosser et al., 1987; Baumrucker and Stemberger, 1989; Winder et al., 1989). It is not known, however, if IGFs and other growth factors have similar roles in intact animals. In order to find insights into this question, the expression levels of IGFs, IGFBPs and IGF receptors in mammary glands and their concentrations in the circulation during pregnancy were determined.

CHAPTER 2

REVIEW OF LITERATURE

Insulin-Like Growth Factors

Historical Perspective

In 1957, Salmon and Daughaday observed that neither growth hormone nor sera from hypophysectomized rats stimulated sulfate uptake by costal cartilage in explant cultures, whereas sera from normal rats or hypophysectomized rats that were subsequently treated with growth hormone stimulated sulfate uptake. From this observation the somatomedin hypothesis was formulated which states that growth hormone stimulates skeletal growth indirectly through generation of an intermediary "sulfation factor." Subsequently, plasma extracts with sulfation activity were found also to have mitogenic and insulin-like activities, which led Daughaday et al. (1972) to coin the term "somatomedin" to define the partially characterized plasma factors which mediated the growth-promoting activities of GH on somatic tissues.

The insulin-like growth factors (IGFs)-I and -II, which, as their names imply, have insulin-like and growth-promoting activities, were purified from human plasma (Rinderknecht and Humbel, 1978a,b). Somatomedins A (Sievertsson et al., 1975) and C (Van Wyk et al., 1974) were isolated from human plasma based on their sulfation and sulfation/mitogenic activities, respectively, and were found to be identical to IGF-I in their respective amino acid sequences (Klapper et

al., 1983; Enberg et al., 1984). Multiplication-Stimulating Activity (MSA), which was isolated from calf serum (Pierson and Temin, 1972) and rat liver conditioned medium (Dulak and Temin, 1973) based on its mitogenic activity, was found to be identical to IGF-II (Marquardt et al., 1981). By convention, somatomedins A and C and MSA are now referred to as IGF-I and IGF-II, respectively (Daughaday et al., 1987a). There are several other partially characterized IGF-like activities in plasma (Dulak and Temin, 1973; van Buul-Offers et al, 1988); however, their exact identities are unknown.

Classically, liver is believed to secrete IGF-I in response to GH, which elicits increased somatic growth (somatomedin hypothesis). The somatomedin hypothesis has been challenged several times during the past decade. Since IGF-I immunoreactivity and the GH dependence of it were found in extracts of various tissues (D'Ercole et al., 1984), the significance of circulating IGF-I was raised. Also contradictory to the somatomedin hypothesis was the demonstration that direct injection of GH into the tibial cartilage growth plate stimulated growth of the ipsilateral growth plate with no effect on the contralateral growth plate (Isaksson et al., 1982). Similarly, the dual effector theory (Green et al., 1985), which was proven to be valid in a few experimental systems, predicted that IGF-I cannot exert its mitogenic actions on undifferentiated (precursor) cells until such cells are induced to differentiate by the action of GH. Recently, it became clear that IGF-I mediates or augments the actions not only of GH but also of other tropic hormones including FSH, TSH and estrogen (see review by Hall and Sara, 1990). However, evidence for the growth-promoting effects of circulating

IGFs in animals and human subjects has accumulated at the same time due largely to the recent availability of recombinant IGFs. The somatomedin hypothesis therefore holds true, but only to a limited extent.

Use of molecular biological techniques has led to rapid advancements in understanding the biosynthesis and mechanism of actions of IGFs. IGF cDNAs have been cloned in several species (see review by Rotwein, 1991). Use of these cDNAs in RNA-DNA hybridization techniques revealed that high levels of IGF mRNAs are expressed in fetal tissues prior to the ontogeny of the GH system (Lund et al., 1986). This supports the autocrine/paracrine theory of IGF action (D'Ercole et al., 1984, 1986; Han et al., 1987a,b). The GH-dependence of IGF expression in hepatic and nonhepatic tissues in adult animals was also demonstrated by these methods (Roberts et al., 1986; Hynes et al., 1987). The intermediary role of IGF-I in the action of GH was substantiated using GH-transgenic mice (Mathews et al., 1988), whereas the suggested role for IGF-II as a fetal growth factor (Moses et al., 1980) was confirmed by gene disruption experiments in mice (DeChiara et al., 1990). At present, nucleotide sequences of IGF chromosomal genes are available for a few species and the transcriptional and post-transcriptional regulatory mechanisms of IGF gene expression are just emerging.

IGF Peptides

IGFs -I and -II are single-chain polypeptides of 70 and 67 amino acids, respectively, consisting of four domains: B, C, A, and D beginning from the NH_2-terminus (Rinderknecht and Humbel, 1978a,b). Domains B and A are structural homologues of the insulin B- and A-chains, respectively. The C-domain of the IGFs corresponds to the connecting (C) peptide of

proinsulin which is not present in mature insulin, whereas the D-domain is an extension of the A-domain and is not present in proinsulin. In addition, the locations of the three disulfide bridges are conserved among the three peptides. The greatest distinction between the two IGFs resides in their dodecylpeptide C-domains. A three-dimensional structure for either IGF molecule has not been determined; however, computer modeling indicated that the IGFs and insulin have similar tertiary structures with minor differences which are related to the specificities of the molecules (Blundell et al., 1978). The structure-function relationships for the IGFs have been partially characterized. Tseng et al. (1987) demonstrated that synthetic hybrid molecules consisting of the B-chain of insulin linked via disulfide bridges to A or A+D-domains of IGF-I had greater affinity for Type I IGF receptors and 3- to 5-fold greater mitogenic potency relative to insulin, with no affinity for IGF-binding proteins (IGFBPs). From this observation, it was concluded that the A-domain of IGF-I is involved in the binding to its receptors and subsequent biological actions, whereas the B-domain of IGF-I is responsible for interaction with IGFBPs. Consistent with this suggestion, des-(1-3)-IGF-I lacking the first three amino acids of the IGF-I B-domain is known to have reduced binding affinities for IGFBPs-1, -2, and 3 (Forbes et al., 1988).

Mature IGF-I is proteolytically generated from IGF-IA and IGF-IB precursor polypeptides (Rotwein et al., 1987a). Pre-pro-IGF-IA and -IGF-IB molecules contain a presumptive 48-residue signal peptide, which is co-translationally cleaved, and a carboxy-terminal extension (E)-domain with 35 and 77 amino acid residues, respectively. Mechanisms by which IGF precursor molecules are processed are not known. Powell et al. (1987)

observed that the IGF-IA pro-peptide (no signal sequence) circulates in patients with chronic renal failure and accounts for ~10% of the total serum IGF-I. In normal serum, however, the IGF-IA immunoreactivity was almost undetectable, which was associated with serum IGF-IA protease activity. In line with this, cultured human fibroblasts secreted IGF only in the pro-IGF form (Conover et al., 1989). These results, taken together, suggest that a substantial proportion of IGF may be secreted in a pro-IGF form(s) which is then proteolytically processed in plasma. Similarly, the E-domain of the IGF-II pro-peptide has been detected in conditioned culture medium of rat BRL-3A liver cells and rat serum (Hylka et al., 1987). It is not known, however, if the circulating E-domain peptides from either pro-IGF has any physiological function.

IGF molecules are evolutionarily well-conserved across the species (see review by Rotwein, 1991). There are only five amino acid substitutions out of 70 amino acids of IGF-I molecules from seven different mammals and three of these five substitutions are conservative changes. When the comparison is extended to 10 species including chicken, xenopus and salmon, 54 amino acids (77%) are invariant. Similarly, IGF-II molecules from six mammals exhibit 89.5% conservation of the 67 amino acids. The growth-promoting activity of IGFs is thus likely to be conserved from fish to mammals in the evolutionary hierarchy.

There are several identified IGF variants. The des-(1-3)-IGF-I variant was isolated from human brain tissues (Sara et al., 1986; Carlsson-Skwirut et al., 1989), bovine colostrum (Francis et al., 1986), and porcine uterus (Ogasawara et al., 1989). This truncated IGF-I is believed to result from a post-translational modification, because the

point of truncation does not correspond to any intron-exon junction of the IGF-I gene. An IGF-II variant which has an Arg-Leu-Pro-Gly sequence in place of Ser-29 was purified from human plasma (Hampton et al., 1989) and was found to correspond to the nucleotide sequence of a variant IGF-II mRNA (Jansen et al., 1985), presumably generated by use of an alternative splice site near the 8th intron-exon junction. This variant IGF-II reportedly comprises ~25% of total IGF-II pool in human plasma and has about one third the potency for binding to the Type I IGF receptors relative to normal IGF-II (Hampton et al., 1989). Zumstein et al. (1985) isolated another IGF-II variant with a molecular mass of 10 kDa which contains a Cys-Gly-Asp tripeptide for Ser-33 and an additional 21 amino acid residues of the E-domain. The substitution of the tripeptide, however, apparently does not result from use of an alternative splice, suggesting the possible existence of an allelic IGF-II gene. Haselbacher and Humbel (1982) described a 9 kDa "big" IGF-II in human spinal fluid which has not been characterized further.

Structures of IGF Genes

IGF-I, as well as IGF-II, is encoded by a single-copy gene (Tricoli et al., 1984; Brissenden et al., 1984) whose nucleotide sequence has been partially determined in the human (Rotwein et al., 1986), rat (Shimatsu and Rotwein, 1987), sheep (Dickson et al., 1991), and chicken (Kajimoto and Rotwein, 1991). The human IGF-I gene spans at least 90 kb with at least five exons. Exon 1 contains 5′ untranslated sequences. Exon 2 encodes the signal peptide and 25 amino acids of the IGF-I B-domain, exon 3 for four remaining B-domain amino acids, the rest of mature IGF-I and 16 amino acids of the E-domain. There is a long intron between exons 2 and

3, the length of which has not been accurately determined. Exons 4 and 5, which are mutually exclusive with respect to RNA splicing, encode 61 and 103 amino acids of the E-domain, respectively. Thus, IGF-IA mRNA which contains exons 1-3 and 5 encodes 195 amino acids, whereas IGF-IB mRNA contains exons 1-4 and encodes a second IGF-I pre-pro-peptide of 153 amino acids (Rotwein, 1986). As a result of this differential splicing, as well as use of variable polyadenylation sites, multiple IGF-I mRNAs with lengths ranging from 0.7 kb to ~8 kb are generated. Recently, Steenbergh et al. (1991) found that 1.1 and 7.6 kb hIGF-I mRNAs transcribed from the same exons (1-3 and 5) are generated by differential polyadenylation. The exon numbering described follows the published literature which differs slightly from the numbering system adopted by workers in the field at a meeting held during the Second International IGF/Somatomedin Symposium in San Francisco on January 12-16, 1991. In that meeting, the previously published exon 1 (see review by Sussenbach, 1989) was divided into exons 1 and 2 with one number shifted for the remaining exons.

Rat and sheep IGF-I genes have the same structural organization as the human IGF-I gene. Sequence analysis of rIGF-I cDNAs has revealed that rIGF-I mRNAs have multiple 5′ untranslated regions (Roberts et al, 1987; Lowe et al., 1987) which are believed to result from alternative promoter usage as well as differential intra-exonic (exon 1) splicing (Rotwein, 1991). There is a species difference in the utilization of exons 4 and 5. In contrast to the mutual exclusiveness of these two exons in the human, in the rat and mouse, exon 4 (52 nucleotides) is either spliced out or fused with exon 5 to generate a frame shift in the exon 5 open reading frame (Bell et al., 1986; Roberts et al., 1987). As a result of the

insertion and frame shift, the E-domains of murine IGF-IA and IGF-IB pre-pro-peptides are totally divergent. The structural organization of the IGF-I gene is different between mammals and avian species. In the chicken, there are 4 exons and the alternative transcriptional initiation and mammalian homologues of IGF-IB mRNAs are not present (Kajimoto and Rotwein, 1991).

The structure of the IGF-II gene has been elucidated in the human (Bell et al., 1985; de Pagter-Holthuizen et al., 1988; Holthuizen et al., 1990), rat (Frunzio et al., 1986; Chiariotti et al., 1988), and mouse genomes (Rotwein and Hall, 1990). The human IGF-II gene (nine exons), localized on chromosome 12, is contiguous with the insulin gene. The first six exons contain noncoding leader sequences and the last three exons coding sequences. The transcript initiated from the P1 promoter, adjacent to 3′ end of insulin gene, is subsequently processed to include exons 1-3 and 7-9 resulting in a 5.3 kb IGF-II mRNA. Promoters P2, P3, and P4 preceding exons 4, 5, and 6, respectively, direct the transcription of the immediately following noncoding exon and the last three coding exons. As such, IGF-II mRNAs with differing lengths are generated through the differential use of transcriptional initiation sites and alternative RNA splicing as well as from use of variable sites of polyadenylation. Interestingly, a 1.8 kb mRNA whose coding sequences are derived from exon 9 of IGF-II gene was translated to yield a 8.3 kDa protein unrelated to IGF-II in the reticulocyte lysate system (de Pagter-Hothuizen et al., 1988). However, the promoter for this RNA transcript and the role of the protein, if translated in vivo, are unknown.

The murine IGF-II gene is also contiguous with the insulin gene, suggesting that the IGF-II-insulin locus was present before mammalian radiation (Rotwein, 1991). The structure of the murine IGF-II gene is homologous to that for the human, except for the absence of leader exons corresponding to the human exons 1-3. As for the human IGF-II gene, only the last three exons encode protein sequence. Murine exons 4 and 5, corresponding to human exons 7 and 8, encode a 24-residue signal peptide, a 67-residue mature IGF-II peptide and the first 11 amino acids of the E-domain. Exon 6 encodes 89 amino acids of the E-domain and also contains 3′ untranslated sequences. There is strong homology between the murine and the human in the nucleotide and amino acid sequences of IGF-II precursors. There are three murine IGF-II gene promoters; no murine promoter homologous to the human P1 promoter has been identified to date. Murine exon 1 contains multiple transcriptional initiation sites, which adds to the diversity of IGF-II mRNAs (Rotwein and Hall, 1990). Rotwein and Hall (1990) also found two regions in the mouse IGF-II gene that are homologous to human exons 2 and 3. These were termed pseudo-exons, since their sequences are not found in mouse IGF-II mRNAs. These pseudo-exons may represent evolutionarily retained remnants of the prototype IGF-II gene.

IGF Expression Tissues

IGF-I gene expression has been reviewed in detail by Simmen (1991). The IGF-I gene is expressed in various fetal tissues prior to maturation of the somatotropic (GH-IGF-I) axis. IGF-I mRNAs were localized by in-situ hybridization to connective tissues or cells of mesenchymal origin in human fetuses (Han et al., 1987a). Using Northern blot hybridization, Han

et al. (1988) detected IGF-I mRNAs with lengths of 0.7, 5.3 and 8.0 kb in a variety of tissues from mid-gestation human fetuses. The overall representation of these mRNAs was variable among tissues, suggesting a tissue-specificity of IGF-I mRNAs expressed. In the rat embryo, the IGF-I mRNA levels increased between days 11 and 13, suggesting a role for IGF-I in organogenesis (Rotwein et al., 1987b). By in-situ hybridization, Bondy et al. (1990) localized IGF-I mRNAs to undifferentiated mesenchymal tissues in the vicinity of sprouting nerves, spinal glia, aggregations of mesenchymal surrounding, and areas of tissue remodeling such as cardiac outflow tract in days 14 and 15 rat embryos. In juvenile rat brain, IGF-I mRNA expression was detected in the olfactory bulb, hippocampus and cerebellum (Werther et al., 1990). In rats, IGF-I mRNA levels were higher in nonhepatic fetal tissues than nonhepatic adult tissues, whereas a reverse situation was evident for the liver (Lund et al., 1986). Adamo et al. (1989) reported a 100-fold increase in hepatic IGF-I mRNA levels during the first 50 days after birth in the rat. In the mouse, hepatic IGF-I mRNA levels exhibited a biphasic temporal pattern; increased up to 2.5 week of age after birth followed by a decline and then a peak at ~day 60 coincident with the onset of puberty (Singh et al., 1991). The postnatal increase in hepatic IGF-I mRNA expression levels was also observed for the sheep (Dickson et al., 1991) and chicken (Serrano et al., 1990; Kikuchi et al., 1991). Circulating IGF-I levels in the chicken were temporally correlated with hepatic IGF-I mRNA levels during the post-hatch period, but not during the embryonic period (Kikuchi et al., 1991). In adult bulls, steers and heifers, Hannon et al. (1991) found that the IGF-I mRNA level was far greater in the liver than in the Sterno-mastoid muscle

and that hepatic IGF-I mRNA levels, serum IGF-I levels and IGF-I peptide production in liver explant cultures were correlated with the amplitudes of GH pulses. From theses results, the authors attributed the rank of growth rates for these sex phenotypes (bull > steer > heifer) to the difference in the cascade of GH pulse amplitude-hepatic IGF-I output-serum IGF-I, each of which exhibited the same rank as for growth rate. They also concluded that the liver is the major site of IGF-I synthesis and secretion. In this regard, the pig appears to be somewhat of a unique species since the IGF-I mRNA level was higher in skeletal muscle than in the liver (Leaman et al., 1990; Grant et al., 1991).

The IGF-I mRNA expression in pig uterus has been extensively characterized (Tavakkol et al., 1988; Letcher et al., 1989; Simmen et al., 1989a, 1990, 1992). The IGF-I content in uterine luminal fluids increases transiently during days 10-12 of pregnancy (Simmen et al., 1989a; Letcher et al., 1989) coincident with the onset of rapid conceptus elongation (Geisert et al., 1982). Endometrial IGF-I mRNA expression and conceptus tissue IGF-I content are also elevated at this period (day 12), whereas conceptus IGF-I mRNA levels and endometrial tissue IGF-I content does not change during days 8-14 of pregnancy. Based on these results, Simmen et al. (1989b) suggested a model for the conceptus-endometrium interaction in early pregnant pig uterus: the uterine endometrium secretes autocrine/paracrine IGF-I and uterine luminal fluid mitogen (Simmen et al., 1988a). By day 12, pig conceptuses secrete estrogen which further stimulates the IGF-I secretion from the endometrium to elicit the growth (elongation) and differentiation of conceptus and endometrium for implantation. In this process, IGF-I has been shown to induce conceptus

aromatase activity (Hofig et al., 1991a) which may lead to augmented secretion of estrogen. In a later study, Simmen et al. (1992) found a correlation between circulating progesterone levels and endometrial IGF-I mRNA levels in cycling and early pregnant pigs, suggesting that progesterone may transcriptionally regulate the expression of IGF-I gene in this tissue.

The IGF-II gene is also expressed in a tissue- and development-specific manner. In the human and rat, IGF-II mRNA levels in tissues are high during fetal life and decline rapidly after birth (Scott et al., 1985c; Lund et al., 1986, Brown et al., 1986). In rats, an exception to this general rule is the brain (choroid plexus and leptomeninges) where IGF-II is expressed continuously to adulthood (Stylianopoulou et al., 1988a). A postnatal decline in hepatic IGF-II mRNA levels was also observed in the mouse (Singh et al., 1991). In the human, the 5.3 kb IGF-II mRNA initiated from the P1 promoter is expressed exclusively in adult liver, whereas P3- and P4-initiated mRNAs are expressed in fetal hepatic and nonhepatic tissues and in adult nonhepatic tissues (Scott et al., 1985c; de Pagter-Holthuizen et al., 1988). The tissue and developmental specificity of the 5.0 kb mRNA derived from the human P2 promoter is not known; however, this mRNA was detected in fetal liver (Holthuizen et al., 1990). There are differences in the regulation of IGF-II gene expression between the human and rat. Whereas the P3 promoter is the most active in the human fetus, in the rat, P3 promoter (equivalent to the human P4 promoter) is the most active (Gray et al., 1987; Han et al., 1988; Sussenbach, 1989). As stated earlier, there is no rat homologue of the human P1 promoter.

The temporal and spatial patterns of expression of IGF-II mRNAs in human and rat tissues have been characterized. IGF-II mRNAs were detected by in-situ hybridization in human embryos at day 18 post-fertilization (Brice et al., 1989). Since the IGF-II transcripts were not detected in the blastocyst in this study, it was concluded that the earliest time of IGF-II mRNA expression in the human embryo occurred between days 5 and 18 post-fertilization. IGF-II mRNA expression in kidney, which was localized to metanephric blastema cells, declined with blastema differentiation during the first trimester, while the reverse temporal trend was apparent in the cytotrophoblast layer of the placenta.

In the rat embryo, IGF-II mRNAs were abundant in tissues of mesodermal origin including developing muscle, cartilage, and blood vessels (Beck et al., 1987; Stylianopoulou et al., 1988b; Bondy et al., 1990). In the study of Stylianopoulou et al. (1988b), levels of IGF-II in chondrocytes were relatively high at mid-gestation and subsequently declined prior to ossification. Yolk sac and liver, among endoderm-derived tissues, exhibited high levels of IGF-II mRNAs, whereas ectoderm-derived tissues, such as the nervous system and skin, were negative by in-situ hybridization in these studies.

Sites of IGF mRNA expression do not always coincide with sites of localization and action of IGF peptides. Han et al. (1987a) found that the tissue sites of IGF-I mRNAs did not correspond to IGF-I immunoreactive sites in early human fetuses. Another good example of this phenomenon is found in the kidney. It is well established that IGF-I is synthesized in the collecting duct, whereas Type I IGF receptors are localized in glomeruli and proximal tubules (Hammerman, 1989). Similarly, cell type

expression of IGF-II mRNAs in human fetal kidney did not spatially correlate with either high mitotic activity or differentiation (Brice et al., 1989). In the adult rat ovary, IGF-I mRNA production is granulosa cell-specific (Oliver et al., 1989), while IGF-II production is limited to theca-interstitial cells (Hernandez et al., 1990). However, mRNAs encoding Types I and II IGF receptors were detected in both types of cells. On the other hand, sites of IGF-I mRNA synthesis in the juvenile rat brain (Werther et al., 1990) corresponded to or were adjacent to sites of Type I IGF receptor expression. Collectively, these results suggest that locally synthesized IGFs act primarily through autocrine and paracrine routes. IGF peptides that escape the tissue sequestration may then enter the general circulation.

IGF Expression in The Mammary Gland

The stromal cell layer appears to be the major tissue site of production of IGFs-I and -II in the mammary gland. By in-situ hybridization (Yee et al., 1988, 1989), IGF mRNAs were detected in the stromal cell layer but not in the epithelial cells of human breast tissues. Consistent with this result, Glimm et al. (1988) localized immunoreactive IGF-I to stromal cells in bovine mammary tissues. There is as yet no evidence for production of IGF-I by epithelial cells. Although Glimm et al. (1988) detected IGF-I immunoreactivity in epithelial cells after GH injection, the epithelial IGF-I may have originated from the stroma. Campbell et al. (1991) reported that immunoreactive IGF-I released from cultured bovine mammary acini was not suppressed by cycloheximide, suggesting that epithelial cells do not synthesize IGF-I de novo. Published results concerning IGF-I production by cultured human

breast cancer cells are inconsistent. Karey and Sirbasku et al. (1988) reported that immunoreactive IGF-I was released from cultured MCF-7 cells, but this result was not supported by the study of van der Burg et al. (1990). Similarly, Baxter (1983) reported that the IGF-I immunoreactivity in conditioned medium of human T47D breast cancer cells had a molecular mass of 35 kDa which did not shift to a lower molecular mass upon acidification, suggesting that the IGF-I immunoreactivity may have represented an artifact due to interference by IGF-binding protein(s).

Growth hormone (GH) appears to be a physiologic regulator of mammary IGF-I expression. Kleinberg et al. (1990) found that GH increased mammary IGF-I mRNA levels in sexually immature, E_2-implanted, hypophysectomized male rats. Interestingly, hGH, which is lactogenic, was more effective than the non-lactogenic rGH, but neither human nor rat prolactin exhibited a mammogenic effect. Consistent with this observation, Glimm et al. (1988) observed increased IGF-I immunostaining in bovine mammary tissues following GH injection. In this regard, the detection of GH receptor mRNAs in bovine and rabbit mammary epithelial cells as well as in the other mammary cell components (Hauser et al., 1990; Glimm et al., 1990; Jammes et al., 1991) further supports the possibility of direct action of GH on the mammary gland. It remains to be determined, however, if the GH receptor mRNAs are translated into functional proteins and if so, what role(s) this receptor may have in this organ.

Both estrogen and progesterone are mammogenic hormones (Knight and Peaker, 1982; Tucker, 1987), but the effects of these steroids on mammary IGF expression are not known. In a study of van der Burg et al. (1990), cultured MCF-7 human breast cancer cells did not release significant

amounts of IGF-I either in the presence or absence of estrogen. This suggests that the mitogenic effect of estrogen in this cell line is not mediated via IGF-I and that the IGF-I immunoreactivity detected in the conditioned culture medium of MCF-7 cells (Huff et al., 1986) was an artifact of IGF-binding proteins.

IGFs are also found in colostrum and milk in various species (Baxter et al., 1984; Malven et al., 1987; Corps et al., 1988; Simmen et al., 1988b, 1991). Although it is generally unclear why hormonal substances are found in mammary secretions (Peaker and Neville, 1991), colostrum-derived IGFs have potential roles in neonatal growth and development. Concentrations of IGFs in colostrum are higher than those for maternal serum, i.e., colostrum contains a physiologically significant quantity of IGFs for delivery to the neonate. The acid-stability of IGFs as well as the high trypsin inhibitor activity in the gut of the neonate renders the delivery of intact IGFs to the pre-closed neonatal intestine physiologically feasible. As a matter of fact, Baumrucker and Blum (1991) found that orally administered IGF-I increased serum IGF-I levels while decreasing serum insulin levels in newborn calves, indicating absorption of administered IGF-I. In this regard, the intestine appears to be a target of dietary IGFs in newborn animals. Schober et al. (1990) found that the rapid intestinal growth of newborn pigs was associated with high levels of Type I IGF receptors, suggesting that colostral IGFs may have a role in gut growth and development. In support of this, Baumrucker et al. (1991a,b) reported that orally administered IGF-I increased intestinal sugar transport capacity, cellular proliferation, and IGF-binding capacity in newborn calves.

Diurnal, Developmental and Pregnancy-Specific Changes in Plasma IGF Levels

Circulating IGF-I levels do not exhibit diurnal variations (Horner et al., 1981; Lee et al., 1991b). Baxter et al. (1983) reported that the serum IGF-I level fluctuated within a two-fold concentration range in a pulsatile manner in adult rats. They observed a 1-1.5 h time lag between the GH pulse and IGF-I pulse and a significant correlation between serum IGF-I levels and integrated GH concentrations (total GH outputs). However, these results appear to be limited to the rat. In beef cattle, serum IGF-I concentrations did not exhibit the pulsatility and were correlated with GH peak amplitudes, but not with total GH outputs (Lee et al., 1991b). The correlation between GH pulse amplitudes and serum IGF-I concentrations in cattle was also reported by Hannon et al. (1991), suggesting that the GH pulse amplitude is a main determinant of IGF-I secretion in cattle.

Circulating IGF-I levels are low during fetal life and increase with postnatal development in all mammalian species so far examined (Zapf et al., 1981; Gluckman and Butler, 1983; Donovan et al., 1989; Bishop et al., 1989; Badinga et al., 1991). Since the opposite temporal trend was apparent in the tissue IGF-I mRNA expression in the rat (Lund et al., 1986), it is generally believed that IGF-I is utilized primarily through an autocrine/paracrine route in the fetus whereas endocrine IGF-I assumes an increased significance after birth (Daughaday and Rotwein, 1989). The postnatal increase in serum IGF-I concentrations in rats is correlated temporally with hepatic GH receptor binding (Maes et al., 1984), as well as hepatic IGF-I mRNA levels (Adamo et al., 1989). In addition, it has been reported that IGF-I outputs from cultured primary hepatocytes or

perfused livers could account for the bulk of the total circulating IGF-I pool in adult rats (Schwander et al., 1983; Scott et al., 1985a,b). These results are consistent with the notion that liver is the major endocrine source of IGF-I. However, except for the temporal correlation between the hepatic GH binding and serum IGF-I concentrations (Gluckman et al., 1983b; Breier et al., 1989; Badinga et al., 1991), data supporting this concept are lacking for large animal species.

The developmental change in serum IGF-I concentrations in humans has been well characterized (Zapf et al., 1981; also see review by Daughaday and Rotwein, 1989). Serum IGF-I concentrations increase slowly during postnatal development until puberty when they increase to twice the adult levels. The pubertal increase in serum IGF-I level is related temporally to the surge of gonadal hormones in humans and cattle (Lee et al., 1990), but in rodents it is independent of gonadal hormones (Siddiqui et al., 1989; see also review by Daughaday and Rotwein, 1989).

Serum IGF-II concentrations in rats are high at birth and rapidly decline to very low levels within 3 week after birth (Moses et al., 1980); hence, IGF-II has been considered primarily a fetal growth factor. The postnatal decline in circulating IGF-II levels in this species is also coincident with decreases in hepatic IGF-II mRNA expression (Brown et al., 1986), which led to the supposition that liver is the primary source of endocrine IGF-II (Sara and Hall, 1990). However, in humans and large animals, IGF-II levels increase after birth (Zapf et al., 1981; Bandinga et al., 1991; Lee et al., 1991a), even though the reverse situation is apparent for hepatic IGF-II mRNA expression in the human. Thus, the major endocrine source of IGF-II in these species remains unknown.

The serum IGF-I levels of pregnant women increase during the last trimester (Gargosky et al., 1990a; Luthman et al., 1991) and this has been attributed to the rise in circulating concentrations of the placental GH variant (Caufriez et al., 1990). In pregnant rats, in contrast, IGF-I levels increase up to days 12-13 and decline by days 16-17 (Gargosky et al., 1990b). Gargosky et al. (1990a) explained this species difference in terms of energy needs for supporting the fetus. According to these authors, rats, but not women, need a protein catabolism during late pregnancy. This was because, they explained, pregnant rats support three times as much fetal tissue as pregnant women on a bodyweight basis and because at term, a rat fetus grows 20 times faster than does a human fetus. Serum IGF-II levels change biphasically in pregnant women (Gargosky et al., 1990a); declining during early pregnancy and then increasing above levels for nonpregnant women after the first trimester.

Regulation of IGF Gene Expression

GH is the main regulator of IGF gene expression. Administration of GH increases the level of IGF-I mRNAs in the liver in normal (Mathews et al., 1986) and hypophysectomized (Hynes et al., 1987; Lowe et al., 1988) adult rats. The response of IGF-I gene expression to exogenous GH in nonhepatic tissues, however, may be dependent on the endogenous GH status of the animal. In normal rats, exogenous GH did not affect IGF-I mRNA levels of most nonhepatic tissues examined, whereas in hypophysectomized rats, nonhepatic IGF-I mRNA levels were increased by GH administration (Lowe et al., 1988; Vikman et al., 1991). Recently, Grant et al. (1991) reported that in pituitary-intact growing pigs, GH injection increased the hepatic IGF-I mRNA levels by 3-fold while nonsignificantly increasing (<

1.5-fold) the IGF-I mRNA levels in the Longissimus Dorsi muscle. However, their results were not conclusive in determining the GH responsiveness of the skeletal muscle, since tissues were removed 24 hour or longer after the last GH injection. It is known that the IGF-I mRNA levels in skeletal muscle are elevated in response to GH in hypophysectomized rats (Isgaard et al., 1989) and that this effect is minimal or nonsignificant at 24 h after the GH injection (Mathew et al., 1986; Haynes et al., 1987; Isgaard et al., 1989). GH reportedly up-regulates hepatic IGF-I gene transcription (Mathew et al., 1986) and may also regulate IGF-I mRNA processing. In the study of Lowe et al (1988), IGF-IB mRNAs were preferentially increased in the liver following GH injection, while in the kidney, lung and heart, IGF-IA and IGF-IB mRNAs were increased proportionately. Circulating IGF-I concentrations in response to exogenous GH were increased or not affected in rats (Murphy and Friesen, 1988; Orlowski and Chernausek, 1988), whereas in large animals, they were consistently increased (Chung et al., 1985; Bass et al., 1991; Vicini et al., 1991). Effects of GH on IGF-II gene expression appear to be tissue-specific. GH did not affect IGF-II mRNA levels in the liver and pancreas (Hynes et al., 1987), but they were increased in the brain (Hynes et al., 1987) and skeletal muscle (Turner et al., 1988).

IGFs exert feedback regulation on the hypothalamus and pituitary to reduce GH secretion (Rosenfeld et al., 1989). It was reported that IGF decreased GHRH release and concomitantly increased somatostatin release by the hypothalamus (Berelowitz et al., 1981; Shibasaki et al., 1986). At the pituitary, IGF reportedly suppresses GH mRNA expression and protein release (Ceda et al., 1987; Namba et al., 1989). In this process, IGF-I

has a greater feedback effect than IGF-II, just as the former is more GH-dependent than the latter.

In addition to GH, other hormonal or regulatory factors are also implicated in the control of IGF gene expression. Adams et al. (1983) have shown that production of IGF-II by rat fetal fibroblasts was increased by placental lactogen and that with maturation of the fetus, fibroblasts switched to IGF-I production which was regulated by GH. This observation may explain, in part, the predominance of IGF-II in the fetal circulation and the postnatal increase in circulating IGF-I levels in this species.

It is well established that hepatic and nonhepatic IGF mRNA expression are depressed under conditions of nutritional deprivation or insulin insufficiency (Leaman et al., 1990). Glucocorticoids have been reported to suppress hepatic and nonhepatic IGF-I mRNA expression in the rat (Luo and Murphy, 1989). IGF expression is also regulated by tropic hormones at corresponding target tissues. TSH has been reported to increase the secretion of IGF-II, but not IGF-I, from cultured FRTL-5 thyroid follicular cells (Maciel et al., 1988). Both FSH and LH are known to stimulate the biosynthesis of IGF-I in the testis and ovary (Chatelain et al., 1987; Hsu and Hammond, 1987; Mondschein and Hammond, 1988). Likewise, parathyroid hormone has been reported to increase IGF-I mRNA levels and secretion of IGF-I in osteoblast-enriched cultures from fetal rat bone (McCarthy et al., 1989). Estrogen stimulated IGF-I mRNA expression in the uterus (Murphy and Friesen, 1988) and IGF-I mRNA and protein production in granulosa cells in the rat (Hsu and Hammond, 1987; Oliver et al., 1989). In the pig, estrogen as well as progesterone up-

regulated uterine steady-state levels of IGF-I mRNAs and IGF-I peptide secretion into the uterine lumen, with the effects of the two steroids being nonadditive (Simmen et al., 1990). IGFs, which are synthesized in response to tropic hormones, mediate or potentiate the actions of those tropic hormones. Certain peptide growth factors are known to stimulate IGF production and/or synergize with IGF. FGF, PDGF and EGF have been reported to stimulate IGF-I production in fibroblast, smooth muscle cell and renal collecting duct cultures, respectively (Clemmons and Shaw, 1983; Clemmons, 1987; Rogers et al., 1991). Although the regulatory mechanisms are not understood, IGF and/or IGF receptor expression have been reported to be elevated in areas of active tissue remodeling such as early pregnant pig uterus (Simmen et al., 1989b), developing blood vessels (Bondy et al., 1990), regenerating tissues or tissues undergoing physically-induced or compensatory growth (Hammerman, 1989; Edwall et al., 1989; Scott et al., 1990; DeVol et al., 1990).

Interactions of IGF and GH: the Dual Effector Theory

Nixon and Green (1984) observed that GH promoted myogenesis as well as adipocyte formation from their corresponding precursor cells that were generated by azacytidine treatment of $10T_{1/2}$ stem cells, whereas IGF-I had no effect on the differentiation of those precursor cells. From this and other observations, Green et al. (1985) proposed the dual effector theory which stated that GH induces the differentiation of preadipocytes and prechondrocytes and then somatomedins (IGFs) promote the clonal expansion of the differentiated adipocyte and chondrocyte cell types. In essence, the GH-priming of precursor cells is a prerequisite for subsequent response of the cells to IGFs in this theory. In support of this theory,

Lindahl et al. (1987a) reported that GH was far more effective than IGF-I in stimulating colony formation by rat epiphyseal cells in culture. These workers also observed that the stimulatory effect of IGF-I on colony formation of epiphyseal chondrocytes isolated from hypophysectomized rats was potentiated when the hypophysectomized rats were pretreated with GH, but not with IGF-I (Lindahl, 1987b). However, results concerning the suggested roles for these two hormones in differentiation of preadipocytes were not consistent. Zezulak and Green (1986) reported that GH stimulated differentiation of preadipose 3T3-F422A cells and that pretreatment of the precursor cells with GH increased their sensitivity to the mitogenic action of IGF-I. In contrast, Smith et al. (1988) demonstrated that IGF-I can stimulate the differentiation of 3T3-L1 preadipose cells completely independent of GH. Collectively, the interactive roles of GH and IGF appear to be variable depending upon the nature of the target cell.

Biological Actions of IGFs

The in vitro biological actions of the IGFs were reviewed previously by this author (Lee, 1988) and are therefore only briefly described here. Actions of IGFs can be classified into three main categories; insulin-like activity, mitogenic activity and the effects on cellular differentiation and/or function. The insulin-like activity of the IGFs includes their stimulation of glucose and amino acid transport and protein synthesis as well as inhibition of lipolysis and protein degradation. The mitogenic action of IGFs has been demonstrated for many cell types.

Genes that are regulated by IGF have been characterized. Zumstein and Stiles (1987) have shown that expression of about 30 genes was regulated by IGF-I in Balb/c 3T3 cells which represented about 0.15% of

the total genes expressed by this cell line. The induction of one subclass of these genes was abrogated by actinomycin D, while the induction of the remaining genes, which appeared to be essential for cell survival, was insensitive to this drug. From these results, it was concluded that IGF-I regulates expression of certain genes (category I) at the transcriptional level and the stability of mRNAs from certain other genes (category II). In rat L6 myoblasts, IGF-I has been shown to induce the mRNA expression of the c-fos proto-oncogene (Ong et al., 1987) and the myogenin gene (Florini and Ewton, 1990), the latter of which was shown to mediate the effect of IGF-I on terminal differentiation including cell fusion and muscle-specific gene expression.

The des-(1-3)-IGF-I variant has been reported to have a 4-fold higher potency relative to intact IGF-I in stimulating DNA synthesis in fetal rat brain cells. This was related to a 1.4-5-fold higher potency of the truncated IGF-I compared to intact IGF-I in displacing radiolabeled IGF-I from its receptors (Carlsson-Skwirut et al., 1989). These authors also observed a lower affinity of des-(1-3)-IGF-I for IGFBPs and proposed that des-(1-3)-IGF-I may represent the local autocrine/paracrine growth factor, while the IGFBP-bound intact IGF-I serves as the endocrine form. The more rapid plasma clearance of injected des-(1-3)-IGF-I demonstrated by Ballard et al. (1991) is consistent with this hypothesis. Gillespie et al. (1990) reported the superior in vivo growth-promoting activity of the truncated IGF-I as compared to the intact IGF-I at a low dose (3 μg daily injection), but not at a high dose (30 μg) in the GH-deficient lit/lit strain of mice. The removal of the tripeptide may expose a receptor binding site on the IGF-I molecule, thereby enhancing its biological actions (Carlsson-Skwirut et al., 1989).

The biological roles for GH and IGF-I suggested by Salmon and Daughaday (1957) have been disputed during the past decade since Isaksson et al. (1982) demonstrated the direct growth-promoting action of GH on the long bone. Eden et al. (1983) subsequently identified GH receptors in the rabbit epiphyseal growth plate, further questioning the validity of the somatomedin hypothesis as originally proposed. Isgaard et al. (1986) showed that local injection of either GH or IGF-I led to increased rat long bone growth. However, GH was more effective than IGF-I in their study and this was interpreted to support the dual effector theory (Green et al., 1985) as well as autocrine/paracrine theory of IGF action (D'Ercole et al., 1984). In line with this suggestion, Scheven and Hamilton (1991) have demonstrated that both GH and IGF-I stimulated thymidine incorporation in vitro by long bone and that this was abolished by addition of a monoclonal antibody to IGF-I to the culture medium. However, the effect of GH on long bone growth (DNA synthesis) was apparent only after at least 5 days in culture. This likely explains why researchers who used shorter duration cultures failed to detect similar effects of GH in the long bone explant cultures. It was also noteworthy that the serum-free medium per se supported growth of long bone, albeit to a reduced extent, which was not abolished by the IGF-I antibody, indicating that not only IGF-I but other autocrine/paracrine growth factors are involved in long bone growth.

The significance of circulating IGF-I to somatic growth has been questioned. Orlowski and Chernausek (1988) have reported that exogenous GH increased weight gain in rats with or without a concomitant increase in serum IGF-I concentrations. However, tissue IGF-I content was

consistently increased in response to GH, suggesting that the tissue pool of IGF-I is more important than the systemic pool of IGF-I in growth promotion by GH. Similarly, Spencer et al. (1991) demonstrated that immunoneutralization of serum IGF-I did not diminish the GH-stimulated weight gain in dwarf mice. However, it has also been reported that administration of IGF-I through an endocrine route can promote growth of neonatal (Philipps et al., 1988), hypophysectomized peripubertal (Schoenle et al., 1985) and normal growing (Hizuka et al., 1986) rats and lit/lit mice (Gillespie et al., 1990). Circulating concentrations of IGF-I have been reported to correlate with body size in miniature poodles (Eigenmann et al., 1984) and with live weights of mice (Blair et al., 1987), indirectly supporting a role for endocrine form of IGF-I in somatic growth.

The suggested role for IGF-II as a fetal growth factor has been experimentally validated. After implanting fetal rat paws under syngeneic kidney capsules and then infusing IGF through the renal artery, Liu et al. (1989) found that both hIGF-I and rIGF-II increased the growth and differentiation of the implanted paws, with rIGF-II being superior to hIGF-I in this regard. IGF-II is thus likely to act through its own receptors, since IGF-II has several-fold lower affinity for the IGF-I receptor relative to IGF-I in the majority of cells and tissues (Massague and Czech, 1982; Rechler and Nissley et al., 1985). DeChiara et al. (1990) have shown that mouse fetal growth was reduced when one allele of the IGF-II gene was inactivated by gene targeting. This is very strong proof for IGF-II's essential role as a fetal growth factor, although the possibility that the reduced fetal growth was secondary to reduced

placental growth (Owen JA, 1991) cannot be ruled out. On the other hand, effects of exogenously administered IGF-II in postnatal rodents were not consistent. A growth-promoting effect of IGF-II was observed in rats that were hypophysectomized at 25-30 days of age and subsequently received IGF-II administration at 50 day of age (Shaar et al., 1989) and also in 6-8 wk old Snell Dwarf mice (van Buul-Offers, et al., 1988). In contrast, in rats that were hypophysectomized at 30-40 days of age and received IGF-II administration 3-4 week after the hypophysectomy, IGF-II was not effective with respect to growth promotion (Schoenle et al., 1985). Nude rats and mice that received transplanted IGF-II-secreting cells exhibited reduced serum IGF-I concentrations and no increased weight gains relative to controls (Wilson et al., 1987). As pointed out in several papers (van Buul-Offers et al., 1989; Humbel, 1990; Sara and Hall, 1990), these negative results must be interpreted with caution, since skeletal tissue responsiveness to IGF diminishes with age and because purities of IGF preparations differ from laboratory to laboratory. Thus, the rapid postnatal decline in IGF-II receptors in rat tissues (Sklar et al., 1989) may, in part, explain the reduced IGF-II effect in the older animals. The substitution of IGF-II for IGF-I in those animals bearing the IGF-II-secreting transplants, which is presumably related to a competition between IGF-I and IGF-II for IGFBP occupation, is also not likely to have any advantage in terms of growth promotion when considered in the context of declining IGF-II receptors. Besides, the effects of exogenously administered free IGFs may be less than those of IGFBP-bound endogenous IGFs due to decreased plasma half-lives or increased clearance rates (Cohen and Nissley, 1976).

In summary, although IGF-I is believed to function primarily as an autocrine/paracrine growth factor, endocrine IGF-I can also promote growth. Likewise, endocrine IGF-II has growth-promoting action, at least in young rats. The lack of a growth-promoting effect of IGF-II in older rodents may be related to the reduced tissue IGF-II receptor abundance. However, these results may not be directly relevant to farm animals, since circulating IGF-II levels in these species increase postnatally whereas they decline in rodents. The involvement of IGF-II in postnatal growth of large animals therefore remains a possibility.

IGFs mediate and/or potentiate the biological actions of tropic hormones. In the ovarian granulosa cells, IGF enhances cellular proliferation (Mondschein et al., 1989) and cytochrome $P450_{scc}$ and adrenodoxin activities in synergy with estradiol (Veldhuis et al., 1986a) and also potentiates FSH-stimulation of aromatase activity (Adashi et al., 1985) and FSH and estrogen-induced progesterone synthesis (Veldhuis et al., 1986b; Davoren et al., 1986). Since IGF expression in these cells is induced by FSH and estradiol (Hsu and Hammond, 1987), the locally synthesized IGFs are likely to act on granulosa cells through Type I IGF receptors (Adashi et al., 1990). In theca-interstitial cells, IGF-I has been found to promote steroidogenesis (Caubo et al., 1989) including LH-induced androgen synthesis (Cara and Rosenfeld, 1988). Similar effects of IGF on luteal cells have been reported (McArdle and Holtorf, 1989; Constantino et al., 1991). Analogously, IGF-I promotes proliferation of FSH-stimulated Sertoli cells (Del Monte et al., 1990) and enhances biological activity of LH on Leydig cells in the rat (Rigaudiere et al., 1989). These effects of IGFs are likely to be elicited in autocrine/paracrine mechanisms <u>in vivo</u>.

In the uterus, IGF-I is believed to mediate estrogen-induced growth. Estradiol reportedly increases uterine IGF-I mRNA expression in ovariectomized, hypophysectomized rats (Murphy and Friesen, 1988) and in immature and mature, ovariectomized pigs (Simmen et al., 1990). Both IGFs-I and -II have been reported to enhance the activities of $P450_{scc}$ and 3ß-HSD while inhibiting the aromatase activity in human placental cytotrophoblasts (Nestler, 1987, 1989, 1990). The actions of the two IGFs in these cells appear to be mediated through their own receptors, since monoclonal antibodies against the Type I IGF receptor did not block the inhibitory effect of IGF-II on aromatase activity (Nestler, 1990). These results contrast with those in the ovary and raise a question as to why IGFs have opposite biological actions in the ovary and placenta, respectively. In a study of IGF-I as a mediator of estrogen biosynthesis (Hofig et al., 1991a), IGF-I was shown to stimulate the aromatase activity in day 12 conceptuses, but not in day 10 pig conceptuses, without affecting overall estrogen production in vitro. These results were interpreted to reflect a requirement of multiple regulatory factors including the IGFs to initiate conceptus estrogen synthesis via an induction of steroidogenic enzymes.

IGF synergizes with TSH at the thyroid gland. Maciel et al. (1988) found that the IGFs potentiated TSH-induced growth of FRTL-5 thyroid follicular cells, as evidenced by the diminution or obliteration of TSH-induced cellular proliferation after immunoneutralization of endogenous IGFs. In a related study, Takahashi et al. (1990) observed that TSH stimulated the production by FRTL-5 cells of factor(s) that potentiated the mitogenicity of IGF-I not only on this cell line but also on human

fibroblasts. This was interpreted to indicate that the mitogenic effect of TSH on FRTL-5 cells was due to its stimulation of production of IGF and the partially characterized competence factor that enhances the responsivity of these cells to mitogenic stimuli. A synergism between TSH and IGF-I in thyroglobulin synthesis by this cell line was also reported (Santisteban et al., 1987).

Biological Actions of IGFs in the Mammary gland

IGF-I stimulates DNA synthesis of ruminant epithelial cells isolated from both undifferentiated and lactating mammary glands (Shamay et al., 1988; Winder et al., 1989). IGF-II was one-tenth as potent as IGF-I in this regard, suggesting that the mitogenic response was via interactions with the Type I IGF receptor. EGF was synergistic with IGF-I in stimulating proliferation of bovine mammary epithelial cells in culture (Shamay et al., 1988). EGF and IGF may act as competence and progression factors, respectively (Stiles et al., 1979), during proliferation of mammary epithelial cells. IGF-I may be essential for the sustained growth of mammary epithelium (Hansen and Knudsen, 1991). These workers defined the minimum hormonal requirements for long-term growth of mammary epithelial cells isolated from lactating goats on collagen gel. Under these conditions, the combination of insulin, hydrocortisone, EGF, T_3, estradiol and bovine serum albumin was minimally required for the sustained cellular proliferation. However, IGF-I could substitute for higher concentrations of insulin. As pointed out by Forsyth (1989), it is likely that the mitogenic action of insulin at high concentrations in many epithelial cell cultures is mediated via the Type I IGF receptor.

The mechanism for the lactogenic effect of GH in ruminants has been of interest to animal scientists (Gluckman et al., 1987), because functional GH receptors have yet to be demonstrated in mammary glands. In the study of Davis et al. (1989), systemic infusion of GH but not IGF-I increased milk yield of lactating goats. Prosser et al. (1990) suggested an answer to the lack of lactogenic response to systemically administered IGF-I. When IGF-I was infused into the pudic artery, milk yield and blood flow were increased significantly in the infused mammary gland, whereas the milk yield from the other side was not increased significantly. According to these authors, the lack of a significant effect of IGF-I on the noninfused mammary gland is attributable to an attenuation of IGF-I by plasma IGF-binding proteins. It thus seems likely that the lactogenic effect of GH is, in part, mediated by IGF-I, although the relative contribution from systemic IGF-I and the substrate-mobilizing effect of GH remains unclear. Results concerning the lactogenic effects of IGF-I in vitro were not consistent. Whereas Baumrucker (1986) reported a stimulatory effect of IGF-I on lactose synthesis by cultured bovine mammary epithelial cells, Shamay et al. (1988) found no effect of IGF-I on α-lactalbumin and fatty acid synthesis. In mouse mammary epithelial cell cultures, IGF-I was 10- to 20-fold less effective than insulin in stimulating synthesis of casein and α-lactalbumin (Prosser et al., 1987).

Assays

Several procedures have been described for assay of IGFs. The assay of sulfate incorporation by cartilage was established before the somatomedin experiments of Salmon and Daughaday (1957). Insulin-like activities are usually measured using fat pads or isolated adipocytes

(Schoenle et al., 1983) and mitogenic activity by thymidine incorporation by cartilage or fibroblasts (Pierson and Temin, 1972; Daughaday et al., 1975). However, these bioassays are not only time-consuming but are relatively nonspecific and, in most cases, have been superseded by specific RIAs. IGFs in biological fluids are, however, bound to IGFBPs which interfere with the interaction of IGF and its antibodies (Furlanetto et al., 1977). To remove the source of interference, various procedures using different acidic treatments have been developed from several laboratories primarily for serum. Daughaday et al. (1980) extracted serum IGFs with acid-ethanol prior to RIA. Acid gel filtration (Zapf et al., 1980), which is still the standard reference method, was used to dissociate and size-exclude IGFBPs from IGF pools. An acid incubation method (Underwood et al., 1982) was used to liberate IGFs from IGFBPs and at the same to denature the IGFBPs. However, all of these procedures are not 100% effective with respect to removal of IGFBP activity. Lee and Henricks (1990) observed that IGF-I concentrations for adult bovine sera after acid-ethanol extraction or acid incubation were underestimated by ~30% relative to those after acid gel filtration or acidic C_{18} reverse-phase HPLC, although the RIA values were correlated among methods. The lower RIA values with the former methods were associated with incomplete removal of IGFBP activity, especially the albumin-size IGFBPs, suggesting that low M_r IGFBPs are resistant to these acidic treatments. The pretreatment method therefore needs to be chosen with caution when dealing with biological fluids that are rich in low M_r IGFBPs, since IGFBP alone can exhibit a false-positive result in the double antibody IGF RIA (Lee and Henricks, 1990; Campbell et al., 1991). It is also known that IGFBPs

in some conditioned culture media are resistant to acid-ethanol extraction (Campbell et al., 1991). For the measurement of IGF content in tissues, IGFs are usually extracted with 1 M acetic acid prior to IGF RIAs (D'Ercole et al., 1984).

Insulin-Like Growth Factor-Binding Proteins

It has been known for many years that IGF activity in plasma exists at a molecular mass of greater than 50 kDa from which it is converted to a low M_r form at low pH (Burgi et al., 1966). This phenomenon was subsequently found to be due to the existence of IGF carrier proteins (Zapf et al., 1975). These workers showed that radiolabeled IGF (NSILA) bound to two protein fractions which appeared in the 50-150 kDa range on gel permeation chromatography. The two fractions (40-60 and 130-150 kDa) were referred to as albumin- and γ-globulin-size complexes in the early literature. Historically, the IGF carrier proteins were regarded as inhibitors of IGF activities. Zapf et al. (1979) observed a reduced bioactivity of NSILA in its bound form relative to the free form and hypothesized that the vascular bed constitutes a diffusion barrier to the IGF carrier complexes. Subsequently, this hypothesis was extended to suggest that the albumin-sized IGF carrier serves as a transcapillary IGF transporter due to its smaller size while the γ-globulin size serves as the major plasma IGF carrier (Zapf et al., 1984). While the Swiss and other groups searched for roles for IGF carrier proteins, a number of important observations were made. The 150 kDa carrier was found to be GH-dependent and saturated with endogenous ligands, whereas the 50 kDa carrier was related inversely to GH status and had unoccupied IGF binding

sites (Moses et al., 1976; Kaufman et al., 1977; White et al., 1981). At the same time, Furlanetto (1980) discovered that the 150 kDa IGF carrier in human serum consisted of an IGF-binding subunit and an acid-labile subunit both of which were GH-dependent proteins. It was demonstrated at about this time that the plasma half-life of IGF was longer when the IGF was associated with the 150 kDa carrier than when associated with the 50 kDa carrier or when in free form (2-4 h vs 8-30 min vs ~10 min, respectively; Cohen and Nissley, 1976). In this regard, the postnatal replacement of the 150 kDa complex for the 50 kDa complex in serum (D'Ercole et al., 1980; White et al., 1982; Butler and Gluckman, 1986; McCusker et al., 1988) suggests that the developmental regulation of serum IGFBP distribution may be related to regulation of IGF action. IGF carriers are now referred to as IGF-binding proteins and are given a numerical designation (Ballard et al., 1990).

IGFBPs en mass have several potential functions (Baxter, 1986; Baxter and Martin, 1989a; Ooi, 1990), although physiological roles for individual IGFBPs are largely speculative. Firstly, IGFBPs are IGF carriers. It was recently demonstrated that IGFBPs-1 and -2 can traverse the capillary endothelium to reach target tissues (Bar et al., 1990). Insulin enhanced the transcapillary movement of IGFBP-1, but not IGFBP-2. The IGFBP-3-containing 150 kDa IGFBP complex, on the other hand, does not appear to readily traverse the endothelium as evidenced by reduced ratio of this complex over total IGFBPs in lymph fluid when compared to serum (Binoux and Hossenlopp, 1988), indicating that IGFBP-3 is the major plasma IGF carrier. These results are consistent with the roles for these IGFBPs as IGF carriers suggested by Zapf et al. (1984). Secondly, IGFBPs may serve

as an IGF reservoir, since there are no known IGF storage sites. The one to two orders of magnitude greater concentration of IGFs in plasma compared to other peptide hormones is consistent with this notion. Thirdly, IGFBPs protect the host from the acute insulin-like activity including the hypoglycemic effect (Zapf et al., 1986). Lastly, IGFBPs are modulators of IGF action at least in vitro. IGFBPs-1, -2, and -3 have both inhibitory and stimulatory effects on IGF-induced responses in vitro, depending on the cell culture conditions (Elgin et al., 1987; Conover et al., 1990; Ooi et al., 1990; Sara and Hall, 1990). As a general rule, IGFBPs were inhibitory to IGF action when IGF and IGFBP were added simultaneously to the culture medium, whereas they were enhancing when the cells were preincubated with IGFBPs prior to addition of IGF. Where IGFBPs were reported to increase IGF activity, IGFBPs were associated with the cell surface thereby facilitating the delivery of IGF to cell surface receptors (Clemmons et al., 1986; Conover et al., 1990).

As for the IGFs, the liver is believed to be the major source of circulating IGFBPs and this appears to be true in the rat. Scott et al. (1985a,b) have reported that the majority of circulating IGF-I and IGF-binding protein (now known as IGFBP-3) is released from the liver in a similar molar ratio as estimated from secretion rates of the proteins from cultured primary hepatocytes from adult rats. Similarly, adult liver has been reported to have the highest level of IGFBP-3 mRNA in rats (Shimasaki et al., 1989). Comparable data for other species are not available.

Insulin-Like Growth Factor-Binding Protein-1 (IGFBP-1)

This class of IGFBP was first identified in human amniotic fluid (Chochinov et al., 1977) and historically has been termed placental

protein 12 (PP 12) (Bohn and Kraus, 1980), pregnancy-associated α1-globulin (α1-PEG) (Bell and Keyte, 1988), BP-28 (Baxter et al., 1987), BP-25 (Lee et al., 1988), or IBP-1 (Brinkman et al., 1988). This protein has been purified from human endometrium (Koistinen et al., 1986; Bell and Keyte, 1988) and amniotic fluid (Povoa et al., 1984a; Koistinen et al., 1987; Baxter et al., 1987; Busby et al., 1988a). The PP12 was, in fact, a misnomer, because endometrial stroma and their decidualized cells are the major sites of IGFBP-1 production in the pregnant primate uterus (Rutanen et al., 1985; Fazleabas et al., 1989; Waites et al., 1990). Interestingly, IGFBP-1 is a major secretory protein of decidualized human endometrium (Bell et al., 1985) and is found in amniotic fluids at levels that are 2-3 orders of magnitude greater than those in maternal sera (Rutanen et al., 1982). Thus, the IGFBP-1 in amniotic fluid may be derived from maternal decidua (Bell and Keyte, 1988). It is not known, however, why primate decidual cells secrete copious amounts of IGFBP-1 which are likely in excess of local IGFs in molar concentration. Perhaps, IGFBP-1 has some unidentified role(s) independent of the IGFs in the primate uterus.

Nucleotide sequence analysis of rat and human IGFBP-1 cDNAs (Brinkman et al., 1988; Lee et al., 1988; Murphy et al., 1990) revealed that IGFBP-1 is a single-chain polypeptide of 234 amino acids with a predicted molecular mass of 25 kDa. Amino acid sequence homology between human and rat IGFBP-1 proteins is 66%. There are 18 conserved Cys residues clustered at the NH_2- and COOH-termini. Among these, Cys-226 has, by site-directed mutagenesis, been implicated to be essential for IGF-binding (Brinkman et al., 1991). Purified hIGFBP-1 exhibits slightly greater

molecular mass than is predicted from its amino acid sequence; 26-31 kDa of molecular mass in the SDS-PAGE under nonreducing conditions (32-36 kDa under reducing conditions) or 35-40 kDa in gel filtration. However, no carbohydrate moieties have been found in purified hIGFBP-1 (Busby et al., 1988a).

IGFBP-1 undergoes post-translational modifications. Busby et al. (1988a) observed a dimeric form of IGFBP-1 in human amniotic fluid which was almost identical to the monomeric form in terms of binding affinity to IGFs and in potentiating the mitogenic effect of IGF-I on porcine aortic smooth muscle cells. The significance of IGFBP-1 dimerization is therefore unknown. Recently, Jones et al. (1991) found that human decidual cells and human HEP G2 hepatocarcinoma cells secreted predominantly a phosphorylated form of IGFBP-1, but that human amniotic fluid and serum contained a large proportion of the nonphosphorylated isoform, suggesting a post-secretory dephosphorylation. Interestingly, phosphorylated IGFBP-1 secreted by HEP G2 cells had 6-fold higher affinity for IGF-1 than the dephosphorylated isoform and did not potentiate the mitogenic activity of IGF-I in contrast to the stimulatory effect of the nonphosphorylated form. It seems plausible that the bioactivity of IGFBP-1 is modulated by phosphorylation and dephosphorylation. Regarding to its ligand specificity, IGFBP-1 isolated from human amniotic fluid (Baxter et al., 1987; Busby et al., 1988a) had similar affinities for both IGFs-I and -II.

The single-copy IGFBP-1 gene has four exons that span 5.2 kb on chromosome 7 in the human (Alitalo et al., 1989; Cubbage et al.,1989). The promoter activity of the hIGFBP-1 gene has been partially

characterized. Using deletion analysis, site-directed mutagenesis and gel mobility shift assays, Suwanichkul et al. (1990) identified the CCAAT box region as the major cis-regulatory element in basal IGFBP-1 promoter activity and liver factor B1 as the major trans-acting factor that stimulates basal IGFBP-1 promoter activity in human HEP G2 hepatocarcinoma cells. Besides HEP G2 cells, the IGFBP-1 mRNA (~1.5 kb) has been detected in the liver and placental membranes but not in nonhepatic tissues in the human (Brinkman et al., 1988), which contrasts with the expression of this mRNA in rat kidney, liver and decidua (Murphy et al., 1990). In the uterus of cycling women, the IGFBP-1 transcript was detected only in late secretory phase endometrium (Julkunen et al., 1990).

Besides serving as an IGF carrier, IGFBP-1 has other possible roles. First of all, this protein has been implicated as a growth inhibitor. Rutanen et al. (1988) showed that human endometrial IGFBP-1 inhibited IGF-I binding to late secretory endometrial membranes. Croze et al. (1990) also suggested that endometrial IGFBP-1 inhibits the mitogenic action of paracrine IGF-I produced from outer stromal and myometrial cells to allow the decidua to differentiate. Serum levels of IGFBP-1 have been reported to increase in children with renal failure and to be inversely related to GH status (Drop et al., 1984; Povoa et al., 1984b). Baxter and Cowell (1987) observed a several-fold variation in serum IGFBP-1 concentrations during the day, but did not observe the negative GH-dependence, and presumed that the negative GH-dependence observed in previous studies may have been related to an experimental error associated with the diurnal rhythm of IGFBP-1 levels. Hepatic IGFBP-1 mRNA and serum IGFBP-1 levels increase after dexamethasone treatment (Luo et al., 1990) or fasting

(Busby et al., 1988b; McCusker et al., 1991). Taken together, the elevation of IGFBP-1 expression and secretion is coincident with a catabolic state. Lewitt and Baxter (1991) suggested a glucose counter-regulatory role for IGFBP-1 based on the negative regulation of IGFBP-1 secretion by glucose in human liver explant cultures (Lewitt and Baxter, 1989), implicating IGFBP-1 as an IGF chelator. In contrast, Clemmons and co-workers (1986) have implicated IGFBP-1 as a potential enhancer of IGF bioactivity. In this regard, Busby et al. (1989) reported that one of the two physicochemical isoforms of IGFBP-1 isolated from human amniotic fluid adhered to cell surfaces and potentiated the mitogenic action of IGF-I. Clemmons and Garner (1990) further characterized this activity of IGFBP-1 by showing that an unidentified factor(s) contained in serum and cerebrospinal fluid was required for IGFBP-1 to potentiate IGF-I responses.

There are several known regulators of IGFBP-1 expression. Progesterone is an established regulator of biosynthesis and secretion of IGFBP-1 in the uterus (Rutanen et al., 1986; Bell et al., 1991). Insulin down-regulates IGFBP-1 expression. There is a consistent inverse correlation between serum IGFBP-1 levels and insulin status in the human and rat (Suikkari et al., 1988; Unterman et al., 1990). It was also reported that insulin suppressed IGFBP-1 expression at the transcriptional level in HEP G2 cells (Powell et al., 1991). A role for GH in the regulation of IGFBP-1 expression is not clear. Although hepatic IGFBP-1 mRNA and/or serum IGFBP-1 levels were inversely related to GH status (GH injection or hypophysectomy) in the rat and pig (Seneviratne et al., 1990; McCusker et al., 1991), Baxter and Cowell (1987) were unable to

find any difference in serum IGFBP-1 levels between normal and GH-deficient children. IGFBP-1 expression is also related to nutritional status. Serum IGFBP-1 levels are consistently elevated during fasting (Busby et al., 1988b; McCusker et al.,1991), suggesting that a serum metabolite(s) may regulate IGFBP-1 synthesis and/or secretion. However, it is not yet clear if this nutritional effect on IGFBP-1 is mediated via altered insulin and/or GH secretion.

Insulin-Like Growth Factor-Binding Protein-2 (IGFBP-2)

IGFBP-2 has been purified from conditioned culture media of Buffalo Rat Liver (BRL)-3A cells (Lyons and Smith, 1986; Mottola et al., 1986), postnatal rat astrocytic glial cells (Olson et al., 1991), and Madin-Darby Bovine Kidney (MDBK) cells (Szabo et al., 1988). Rat and human IGFBP-2 proteins have 270 vs 289 amino acids, 29.5 kDa vs 31.3 kDa of molecular mass, and over 85% homology as determined from the IGFBP-2 cDNAs (Margot et al., 1989; Brown et al., 1989; Binkert et al., 1989). This difference in molecular mass accounts for the smaller estimated smaller molecular mass for rIGFBP-2 compared to hIGFBP-2 (~30 kDa vs 34 kDa) in ligand blotting experiments (Yang et al., 1989; Roghani et al., 1991). Purified rat and bovine IGFBP-2 exhibit 34-36 kDa (31.5-33 kDa, nonreducing conditions) and 40 kDa of molecular mass under reducing conditions, respectively. In competitive binding, this protein has a higher affinity for IGF-II than for IGF-I, by an order of magnitude, when IGF-II is used as a radioligand, but exhibits equal affinity for both ligands when IGF-I is used as the radioligand (Szabo et al., 1988; Roghani et al., 1991).

IGFBP-2 mRNA is expressed in liver and other tissues with the highest level in the fetal liver in rats (Brown et al., 1989). In all rat tissues

examined, IGFBP-2 mRNA levels decline with postnatal development (Brown et al., 1989; Orlowski et al., 1990), which was also observed in the liver of rhesus monkeys (Liu et al., 1991).

Plasma IGFBP-2 levels have been reported to decrease during fasting or GH deficiency but not to change in response to meal or glucose infusion in the human (Clemmons et al., 1991). Similarly, an increase in serum IGFBP-2 levels during a state of negative energy balance or after hypophysectomy was observed in lactating cows (Vicini et al., 1991), growing pigs (McCusker et al., 1991) and newborn rats (Orlowski et al., 1990). Thus, GH appears to negatively regulate IGFBP-2 synthesis and/or secretion. This is also supported by the observed decrease in serum IGFBP-2 levels following GH injection in cows and pigs (Vicini et al., 1991; Coleman and Etherton, 1991), although IGFBP-2 levels were not suppressed in acromegalic humans (Clemmons et al., 1991). It is not known, however, at what control point GH exerts a negative effect on IGFBP-2 secretion. Boni-Schnetzler et al. (1990) reported that GH did not suppress IGFBP-2 mRNA expression in cultures of primary hepatocytes from adult rats. However, insulin reduced IGFBP-2 mRNA levels in this system, suggesting that insulin may be a primary regulator of hepatic IGFBP-2 gene expression, or that hepatic responsiveness to GH, with respect to IGFBP-2 mRNA expression, may become reduced with postnatal development. In contrast to GH and insulin, IGF-I appears to up-regulate overall IGFBP expression. Camacho-Hubner et al. (1991) have shown that IGF-I increased the abundance of serum 29 kDa and 34 kDa IGFBPs, as well as IGFBP-3, in GH-deficient transgenic mice, suggesting that IGF-I controls the expression of IGFBPs-2 and -3 independently of GH.

Insulin-Like Growth Factor-Binding Protein-3 (IGFBP-3)

IGFBP-3 is the acid-stable IGF-binding subunit of the 150 kDa IGF-IGFBP complex (Furlanetto, 1980). This protein has been purified from human, rat, pig and cow sera and from porcine follicular fluid (Baxter et al., 1986; Baxter and Martin, 1987; Zapf et al., 1988; Walton et al., 1989; Ui et al., 1989; Conover et al., 1990). Mature hIGFBP-3 has 264 amino acids (28.7 kDa) and carbohydrate moieties accounting for an increase of 20-30 kDa in molecular mass as estimated by SDS-PAGE (Baxter et al., 1986; Wood et al., 1988). Rat and porcine IGFBP-3 have 265 and 266 amino acids with 83% and 85% sequence homology with hIGFBP-3, respectively (Shimasaki et al., 1989; Albiston and Herington, 1990; Shimasaki et al., 1990a). In competitive binding, IGFBP-3 exhibits similar affinities for IGF-I and IGF-II (Martin and Baxter, 1986; Baxter and Martin, 1987; Walton et al., 1989). In terms of IGF transport capacity in human plasma, IGFBP-3 concentrations are reportedly equimolar with total IGF concentrations (Baxter and Martin, 1986). This suggests that IGFBP-3 is saturated with its IGF ligands and that IGFBP-3 is the major IGF carrier in the circulation of adults.

The human acid-labile subunit (ALS) of the 150 kDa complex has been purified and characterized by Baxter and co-workers (Baxter, 1988; Baxter and Martin, 1989b; Baxter et al., 1989). As its name implies, this protein irreversibly loses its activity below pH 4.5 (Furlanetto, 1980; Baxter, 1988). Purified ALS exists as two glycosylation variants with 84 and 86 kDa of molecular mass, under both reducing and nonreducing conditions, which diminishes to 50-70 kDa after N-glycanase treatment. The ALS glycoprotein binds IGF-I-IGFBP-3 and IGF-II-IGFBP-3 with equal

affinity to form 140 kDa (major) and 90-12- kDa (minor) complexes, but not free IGF or IGFBP-3. Excess ALS circulates in human plasma in free form, whereas the majority of IGFBP-3 is bound in the 150 kDa complex (Baxter, 1988). The excess ALS secretion may constitute a mechanism for maintaining all IGFBP-3-IGF in the 150 kDa complex, thereby inhibiting free movement of IGF across the capillary endothelium. In this regard, IGFBP-3 and ALS appear to be regulated by different mechanisms. Zapf et al. (1989) reported that exogenous IGF-I increased serum IGFBP-3 levels with no effect on the level of the 150 kDa complex, whereas GH restored the level of the 150 kDa complex in hypophysectomized rats. This suggests that GH may regulate ALS production directly and IGFBP-3 production indirectly via IGF.

In early studies, IGFBP-3 was also called the GH-dependent IGF-binding protein. As for IGF-I, serum IGFBP-3 levels or hepatic IGFBP-3 secretion in vitro is GH-dependent, i.e., decreased in GH deficiency and elevated in acromegaly or following GH administration (Scott et al., 1985b; Baxter and Martin, 1986; Baxter and Cowell, 1987; Walton and Etherton, 1989). Luo and Murphy (1990) found that dexamethasone increased hepatic IGFBP-3 mRNA abundance and serum IGFBP-3 levels in juvenile rats and suggested increased IGFBP-3 secretion as a potential mechanism for glucocorticoid-induced growth retardation.

The IGFBP-3 mRNA is expressed at highest levels in the liver in adult rats, suggesting that this organ is likely the major source of serum IGFBP-3 in this species. However, the hepatic IGFBP-3 mRNA level was not greater than those in ovary and testis of adult pigs (Shimasaki et al., 1990a). Although these authors attributed the low hepatic IGFBP-3 mRNA

level to an underloading for the liver RNA preparation based on a ß-actin control hybridization, this interpretation was not clear since liver is known to have a lower level of ß-actin mRNA per unit weight of total RNA as compared to other tissues (Slagboom et al., 1990). It is, therefore, uncertain if liver is the major source of circulating IGFBP-3 in nonrodent species.

As for the role of IGFBP-3 at the tissue level, two conflicting suggestions have been proposed. Gopinath et al. (1989) observed an inhibitory effect of pIGFBP-3 on IGF-I binding to porcine aortic smooth muscle cells, whereas Conover and Powell (1991) observed a blocking effect of bIGFBP-3 on IGF-I-induced receptor down-regulation in cultured bovine fibroblasts. Although these two observations are apparently unrelated to each other, they converge with respect to the notion that locally acting IGFBP-3 may serve to buffer IGF action by restricting IGF access to the cell. In contrast, Ernst and Rodan (1990) have suggested that IGFBP-3 augments local IGF-I action based on observations with osteoblastic cell lines. In their study, IGF-I was more potent than [QUAYL]IGF-I, a mutated IGF-I with reduced affinity to IGFBPs, in their mitogenic action on an IGFBP-3-secreting cell line. However, IGF-I and [QUAYL]IGF-I exhibited equal mitogenic potencies in another cell line which does not secrete IGFBP-3.

New Insulin-Like Growth Factor-Binding Proteins

IGFBPs-4, -5 and -6 have been purified and their cDNAs cloned by Ling and his co-workers as well as by Zapf's group (see below). There is a discrepancy in the literature between these two groups in the numerical designation for these newly isolated IGFBPs. Here, the designation by

Ling's group was adopted in honor of their first isolation of and cDNA cloning for IGFBP-4. In brief, Ling's IGFBPs-4, -5, and -6 correspond to Zapf's IGFBPs-5, -6, and -4, respectively.

IGFBP-4 has been purified from rat and human sera (Shimonaka et al., 1989; Kiefer et al., 1991a), conditioned media of human TE89 osteosarcoma cells (Mohan et al., 1989) and rat B104 neuroblastoma cells (Ceda et al., 1991), and porcine follicular fluid (Shimasaki et al., 1991b). This is a glycoprotein consisting of 233 and 237 amino acids for the rat and human, respectively, with 30 kDa of molecular mass under nonreducing conditions (32-36 kDa, reducing) which is reduced to 24 kDa upon N-glycanase treatment (Shimonaka et al., 1989; Kiefer et al., 1991a). IGFBP-4 is the second most abundant IGFBP in adult rat serum (Shimonaka et al., 1989). Liver appears to be the major source of this IGFBP in the human and rat. The IGFBP-4 mRNA was most abundant in the liver in juvenile rats, although other tissues expressed this mRNA at low levels (Shimasaki et al., 1990b). A high level of IGFBP-4 mRNA was also observed in adult human liver, with low levels in fetal liver, osteosarcoma cells, and brain (Kiefer et al., 1991a). IGF appears to be involved in the regulation of biosynthesis of IGFBP-4. Ceda et al. (1991) reported that IGF-I increased the secretion of IGFBP-4 as well as IGFBP-3 and a 29 kDa IGFBP by rat B104 neuroblastoma cells, whereas IGF-II suppressed IGFBP-4 secretion while increasing secretion of IGFBP-3 and the 29 kDa IGFBP. However, neither IGF elicited changes in steady-state levels of IGFBP-4 mRNA, suggesting that the IGFs may regulate synthesis and/or secretion of this protein at the post-transcriptional level(s).

IGFBP-5 has been purified from conditioned medium of human U-2 osteosarcoma cells (Andress and Birnbaum, 1991), adult rat serum (Shimasaki et al., 1991a) and porcine follicular fluid (Shimasaki et al., 1991b), and its cDNAs have been cloned from a human osteosarcoma cDNA library (Kiefer et al., 1991b) and rat ovary and human placental cDNA libraries (Shimasaki et al., 1991a). Rat and human IGFBP-5 consist of 272 amino acids (28.5 kDa) with no potential N-glycosylation sites and exhibit 97% amino acid sequence homology (Shimasaki et al., 1991a). Unlike IGFBPs-3 and -4 mRNAs, IGFBP-5 mRNA was expressed ubiquitously with the liver and kidney showing the lowest and highest levels, respectively, among several rat tissues examined, which suggested that IGFBP-5 may serve primarily to regulate local IGF action (Shimasaki et al., 1991a). Biologically, this protein has been reported to enhance IGF-I-stimulated proliferation of human U-2 osteosarcoma cells (Andress and Birnbaum, 1991).

IGFBP-6 was purified from human cerebrospinal fluid (Roghani et al., 1989), porcine follicular fluid (Shimasaki et al., 1991b), human and rat sera (Zapf et al., 1990, Shimasaki et al., 1991a), conditioned media of human He[39] lung fibroblasts (Forbes et al., 1990), SV40-transformed human AG 2804 fibroblasts (Martin et al., 1990) and human U-2 osteosarcoma cells (Andress and Birnbaum, 1991). Mature human and rat IGFBP-6 have 213/216 vs 201 amino acids and 23 kDa vs 21.5 kDa of predicted molecular mass, respectively, with no carbohydrate residues (Kiefer et al., 1991a; Shimasaki et al., 1991b). In the ligand blot analysis of human serum, IGFBP-6 is resolved at a molecular mass of 28-30 kDa just below IGFBP-4 (30 kDa) (Zapf et al., 1990; Kiefer et al., 1991a). Like IGFBP-2, IGFBP-6

has preferential affinity for IGF-II (Forbes et al., 1990; Kiefer et al., 1991a; Roghani et al., 1991). According to Roghani et al. (1991), IGFBP-6 has the highest affinity for IGF-II among the known IGFBPs. In competitive binding studies performed by these workers, IGF-II was 70- and 3-fold more potent than IGF-I when IGF-II and IGF-I were used as radioligands, respectively. It is also known that IGFBPs-2 and -6 are the most abundant IGF carriers in cerebrospinal fluid which correlates with the relatively high IGF-II concentration in this fluid (Haselbacher and Humbel, 1982). Another interesting feature of IGFBP-6 is that a 1.3 kb IGFBP-6 mRNA was expressed ubiquitously with no predominant tissue site of expression in adult rats, suggesting a general role for this protein as a local modulator of IGF action. In the human, the IGFBP-6 mRNA (1.1 and ~2 kb) levels were high in the adult liver and brain and low in fetal liver (Kiefer et al., 1991a).

In summary, six distinct IGFBPs have been purified, their cDNAs cloned, and their genes mapped to human chromosomes 7, 2, 7, 17, 5, and 12 for IGFBPs-1, -2, -3, -4, -5, and -6, respectively (Shimasaki et al., 1991a,b). Among the IGFBPs, only IGFBPs-1 and -2 have near the COOH-terminus the Arg-Gly-Asp sequence motif which is required for binding to cell surface integrin receptors (Hynes, 1987). Eighteen Cys residues clustered in the NH_2- and COOH-termini are well-conserved, although hIGFBP-6 and rIGFBP-6 lack 2 and 4 of these Cys residues, respectively. Perhaps, the conserved Cys residues are involved in forming a ligand-binding pocket. IGFBP-3 is a component of the serum 150 kDa IGF-IGFBP complex, whereas all of the other IGFBPs comprise the 50 kDa IGFBP peak as resolved by gel chromatography. The constituent IGFBPs of the 50 kDa complex, as

well as a truncated IGFBP-3 (Yang et al., 1989), are resolved at molecular mass of 24 kDa and 28-30 kDa by ligand blotting of human serum. However, the relative abundance of each class of IGFBP in the 28-30 kDa range is not known, although the 24 kDa band likely represents a deglycosylated form of IGFBP-4.

Besides these six well characterized IGFBPs, there are other less characterized IGFBPs. Bautista et al. (1991) isolated a unique 29 kDa IGFBP from human femoral bone whose NH_2-terminal amino acid sequence was not identical to that of any known IGFBP. This protein, tentatively named human bone-derived IGFBP (hBD-IGFBP), exhibited several-fold higher affinity for IGF-II than for IGF-I, and had mitogenic activity independent of IGF on mouse osteoblasts and also enhanced IGF-II-stimulated mitogenesis. Bar and coworkers (Bar et al., 1989; Booth et al., 1990) isolated and partially characterized an IGFBP secreted by cultured bovine periaortic endothelial cells. This protein has been reported to contain IGFBP-2 immunoreactivity, but has not been sequenced.

IGFBP Expression in Mammary Cells

IGFBPs have been reported to be secreted from cultured bovine mammary acini and explants (Campbell et al., 1991) and from clonal bovine mammary epithelial cell lines (Skaar et al., 1990). Both insulin and IGF-I increased the secretion of IGFBP by clonal mammary epithelial cells, whereas cortisol, prolactin and GH had no effect on IGFBP secretion. The sizes of secreted IGFBPs were variable depending on the culture system, but were similar to those for serum IGFBPs. However, it is not clear if IGFBPs produced by mammary cells are the same as serum IGFBPs. The IGFBPs from conditioned culture media of bovine mammary explants after acid-

chromatography exhibited comparable affinities for both ligands when IGF-I was used as rediolabeled ligand, but exhibited no competitive affinity for IGF-I when radiolabeled IGF-II was used (Campbell et al., 1991). The IGFBPs appear to be synthesized de novo by mammary epithelial cells as evidenced by the suppression of IGFBP secretion by cycloheximide in acini cultures. Taken together with the stromal IGF production (Glimm et al., 1988), IGFBPs produced by epithelial cells may serve to sequester paracrine IGFs for local use. IGFBPs are also secreted from cultured human breast cancer cells (Baxter et al., 1983; Clemmons et al., 1990). Estrogen receptor-negative cells secreted IGFBPs-1 and -3 and a 24 kDa IGFBP, whereas estrogen receptor-positive cell lines secreted predominantly IGFBP-2 and the 24 kDa IGFBP whose secretion was specifically enhanced by estrogen (Clemmons et al., 1990). It remains to be determined whether estrogen has a similar effect in vivo. Like the IGFs, IGFBPs are also found in mammary secretions (Simmen et al., 1988b; Skaar et al., 1991).

Local Regulation of IGFBP Expression

In general, IGFs induce secretion of their binding proteins. This inductive effect of ligand has been documented in vitro for fibroblasts (Hill et al., 1989), osteoblasts (Chen et al., 1991), neuroblasts (Ceda et al., 1991), and pituitary cells (Simes et al., 1991), although a selective suppression of IGFBP-4 by either or both of the IGFs was also reported for rat neuroblasts (Ceda et al., 1991) and human fibroblasts and epidermal cells (Neely and Rosenfeld, 1992). The IGFBP-inducing activity of IGFs is apparent in vivo as well (Zapf et al., 1989; Camacho-Hubner et al., 1991).

In addition to the IGFs, hormones and growth factors are also involved in the regulation of IGFBP expression. FSH has been reported to inhibit IGFBP secretion from cultured ovarian granulosa cells and Sertoli cells (Adashi et al., 1990; Smith et al., 1990), whereas IGF-I increased IGFBP-3 secretion (Smith et al., 1990). EGF and TGF-ß were stimulatory and inhibitory, respectively, to IGFBP secretion from cultured porcine granulosa cells (Mondschein et al., 1990). Although the FSH-induced down-regulation of IGFBP secretion has been suggested as a mechanism for enhancing gonadotropin and estrogen-induced IGF action implicating IGFBP as an inhibitor of IGF action, this does not explain the induction of IGFBP by its ligand. Rather, IGFBP expression in gonadal tissue is likely regulated through complex interactions of gonadotropin, IGF and other growth factors. Apart from hormonal regulation, cell type-specific IGFBP expression has been reported for the rat ovary. Nakatani et al. (1991) reported that IGFBPs-2, -3 and -4 mRNAs were expressed by interstitial cells, corpora lutea, and atretic granulosa cells, respectively. Interestingly, IGFBP-1 mRNA was not detected in either rat (Nakatani et al., 1991) or porcine (Shimasaki et al., 1991b) ovary, although all of the other known IGFBPs(-2-6) and their mRNAs were found in porcine ovary.

In rat osteoblastic cells, both IGF-I and vitamin D_3 have been reported to increase IGFBP secretion (Chen et al., 1991). Dexamethasone was inhibitory to IGFBP secretion in this cell type which contrasts with the stimulatory effect of dexamethasone on IGFBP secretion *in vivo* (Luo and Murphy, 1990), suggesting a tissue-specific effect of glucocorticoid on IGFBP secretion. The progesterone-dependence of IGFBP-1 secretion from secretory endometrium has been well-documented in primates (Rutanen et

al., 1986; Bell et al., 1991). Recently, IGFBPs-2 and -3 secretion from cultured uterine endometrial stromal cells was found to be enhanced by estrogen plus progesterone (Giudice et al., 1991) which contrasts slightly with the progesterone-dependence of IGFBP-1 secretion.

IGFBP-2 mRNA expression in uteri of farm animals has been characterized (Simmen et al., 1989b; Simmen et al., 1990; Geisert et al., 1991; Simmen et al., 1992). In gilts, IGFBP-2 mRNA levels in uterine endometrium were higher than those for liver (Simmen et al., 1992) and were increased in ovariectomized gilts in response to progesterone administration (Simmen et al., 1990). In cycling gilts, endometrial IGFBP-2 mRNA abundance paralleled circulating concentrations of estrogen (Simmen et al., 1992). Since IGFs are secreted into the uterine lumen of the pig, cow and sheep (Simmen et al., 1989a; Letcher et al., 1989; Geisert et al., 1991; Ko et al., 1991), IGFBP-2 is likely to serve as a carrier/modulator of secreted IGFs. The endometrial IGFBP-2 mRNA expression in the cow appears to be regulated by progesterone, which was suggested from high levels of the mRNA during the luteal phase compared to earlier stages (Geisert et al., 1991). Recently, the IGFBP-2 mRNAs were detected in sheep and horse endometria (M. T. Moser and F. A. Simmen, unpublished results).

Diurnal, Developmental, and Pregnancy-Related Changes in Serum IGFBPs

Plasma IGFBPs-2 and -3 levels in humans do not exhibit diurnal variations (Baxter and Cowell, 1987; Clemmons et al., 1991), whereas IGFBP-1 levels increase during the sleep phase and decrease after meals (Baxter and Cowell, 1987). It thus appears that IGFBPs-2 and -3 are relatively stable forms of plasma IGF carriers, whereas IGFBP-1 may be a

principal trans-capillary IGF transporter under acute control by serum metabolites and/or insulin (Bar et al., 1990; Clemmons et al., 1991).

Of the known IGFBPs, IGFBP-2 is the most abundant in fetal plasma and is low in adult plasma as determined by RIA or ligand/immunoblotting (Romanus et al., 1986; Hardouin et al., 1987; Yang et al., 1989; Lee et al., 1991a, McCusker et al., 1991). This is consistent with the postnatal decline of IGFBP-2 mRNA levels in hepatic and nonhepatic tissues in the rat (Brown et al., 1989; Orlowski et al., 1990). Plasma IGFBP-3 levels are low during fetal life and increase during postnatal development (Baxter and Martin, 1986; Donovan et al., 1989; Lee et al., 1991a; Liu et al., 1991). The major plasma IGF carrier is, therefore, shifted from IGFBP-2 to IGFBP-3 during development. This may implicate an increased significance of endocrine IGF in postnatal vs prenatal animals, since plasma IGFs are more stable when associated with the IGFBP-3-containing 150 kDa complex as opposed to the 50 kDa form (Cohen and Nissley, 1976). The postnatal increase in circulating levels of IGFBP-3 has been reported to correlate temporally with an increase in hepatic IGFBP-3 mRNA abundance in the rhesus monkey implicating the liver as the major source of this IGFBP (Liu et al., 1991). However, the hepatic IGFBP-3 mRNA level for the late fetal monkey was higher than the peak level for postnatal monkeys in this study. As stated in the introduction to this chapter, it is not clear if liver is the major source of plasma IGFBP-3 in nonrodent species. IGFBP-1 is a minor component of plasma IGFBPs in both fetal and adult sera. The plasma IGFBP-1 level has been reported to decline during postnatal development in the human (Drop et al., 1984) which is consistent with the temporal pattern of hepatic IGFBP-1 mRNA expression (Brinkman et

al., 1988), suggesting that liver may be the major source of plasma IGFBP-1 in the human. The postnatal decline of serum IGFBP-1 levels was also observed for the pig (McCusker et al., 1991).

Serum IGFBP-3 is known to undergo proteolytic degradation during the latter half of pregnancy with little or no change in the low M_r IGFBPs in women (Giudice et al., 1990; Hossenlopp et al., 1990). The pregnancy-associated decline in serum IGFBP activity is also apparent for the rat (Gargosky et al., 1990b; Donovan et al., 1991). In this species, not only IGFBP-3, but also IGFBPs-2 and -4 were affected which was also attributed to the action of serum proteases, since hepatic levels of mRNAs encoding IGFBPs-2, -3 and -4 did not decrease during pregnancy (Donovan et al., 1991). In fact, tissue levels of IGFBP-2 mRNA have been reported to increase near term (Margot et al., 1989; Powell et al., 1991). The pregnancy-associated reduction in serum IGFBP activity may accelerate the delivery of IGFs to tissues (Hossenlopp et al., 1990), although the physiological significance of this is not clear at present. On the other hand, plasma immunoreactive IGFBP-1 levels have been reported to remain constant during the latter half of pregnancy in women (Luthman et al., 1991). The IGFBP-1 levels, as determined by ligand blotting, also did not change in rats during pregnancy, although the hepatic IGFBP-1 mRNA level increased (Donovan et al., 1991).

Assay of IGFBPs

Various methods for measuring IGF-binding activity have been reviewed by Baxter and Martin (1989a). In early experiments, biological fluids were incubated with radioligand and then subjected to gel chromatography to identify the size of IGFBPs. In competitive binding assays, the bound

form of IGFBP is separated from free tracer by absorbing it with charcoal (Baxter et al., 1983; Scott et al., 1985a), or the tracer-bound IGFBP is precipitated using a specific antibody or polyethylene glycol (Baxter and Martin, 1986; McCusker and Clemmons, 1988). Affinity-labeling coupled with SDS-PAGE is frequently used to determine the M_r of IGFBP. It is mainly the unoccupied IGFBPs that are detected by these methods, unless the endogenous IGFs are first removed by acid chromatography prior to assay. Immunological techniques (blotting and/or precipitation) are used in combination with affinity-labeling to identify a specific IGFBP. Specific RIAs for IGFBPs-1, -2, and -3 have been described, but these are limited to several laboratories.

At present, ligand blotting (Hossenlopp et al., 1986) is the most widely used method for the determination of IGFBPs for several reasons. First, the size and relative abundance of multiple IGFBPs can be determined simultaneously. Second, competition between various IGFBPs that can occur in competitive binding assays is avoided, since the IGFBPs transferred to the blotting filter are probed with an excess of tracer. Results obtained by ligand blotting usually agree with those from RIAs or follow predictions based on physiological conditions such as GH and nutritional status. However, the relative autoradiographic intensity for each IGFBP class may not be equal to the molar concentration of that IGFBP. Bicsak et al. (1990) found that the abundance of serum IGFBP-3 was underestimated by ligand blotting. According to their data, only the ~40 kDa IGFBP was detected by ligand blotting, although a major proportion of IGFBP-3 immunoreactivity resided at >84 kDa implicating an incomplete dissociation of the 150 kDa IGF-IGFBP complex during SDS-PAGE. They also

found that the dissociation of the 150 kDa complex was increased by boiling or acid treatment of serum prior to SDS-PAGE, but was not complete after either treatment. Taken together, ligand blotting must be considered, at best, a semi-quantitative method.

Insulin-Like Growth Factor Receptors

Type I IGF (IGF-I) Receptors

Type I IGF receptors have a disulfide-linked α-β-β-α tetrameric subunit structure which is homologous to that for the insulin receptor (Massague and Czech, 1982). Nucleotide sequence analysis of a human Type I IGF receptor cDNA revealed that the pro-receptor of 1367 amino acids is cleaved into a 30-residue signal peptide, a 706-residue α-subunit with a predicted molecular mass of 80.6 kDa, a tetrapeptide Arg-Lys-Arg-Arg at positions 707-710, and a 627-residue β-subunit with a predicted molecular mass of 71 kDa (Ullrich et al., 1986). Mature α- and β-subunits are glycoproteins having molecular mass of 135 kDa and 90 kDa, respectively. The α-subunit is an extracellular protein with a Cys-rich (24 Cys) region between residues 148 and 302 and 11 potential N-linked glycosylation sites. The β-subunit is composed of an extracellular domain (residues 711-905), a transmembrane domain having a 24 amino acid hydrophobic sequence, and an intracellular tyrosine kinase domain between residues 973 and 1229 with a potential ATP-binding site (Gly 976 to Gly 981 and Lys 1003). There is a high degree of amino acid homology between the Type I IGF and insulin receptors. The highest homology (84%) is found in the β-subunit cytoplasmic region defining the enzymatic domain for tyrosine kinase activity followed by a region flanking the Cys-rich subdomains of

the α-subunit (64-67%) and an interphase region between the transmembrane and kinase domains of the ß-subunit. The similarity between the two receptors is relatively low at the Cys-rich region (48%), suggesting that this region is likely involved in defining the ligand-binding specificity of each receptor. In fact, Gustafson and Rutter (1990) found that ~55 amino acids of the Cys-rich region of the IGF-I and insulin receptors determined the ligand binding specificity in chimeric IGF-I-insulin receptors, although other COOH-terminal regions of both α-subunits contributed to the receptor specificity. Recently, Zhang and Roth (1991) reported that a chimeric insulin receptor bearing the Cys-rich region of the IGF-I receptor (residues 184-286) substituted at the corresponding region in the insulin receptor (residues 191-297) exhibited high affinity binding to both IGF-I and insulin. Thus, the Cys-rich region is the determinant for ligand specificity, at least in the IGF-I receptor.

There are several apparent subtypes of Type I IGF receptors (Rechler and Nissley, 1985). Morgan and Roth (1986) found that a monoclonal antibody (5D9) inhibited insulin and IGF-I binding to their corresponding receptors in human IM-9 lymphoid cells and solubilized placental receptor preparations, whereas this antibody inhibited insulin but not IGF-I binding to human HEP G2 cells. Alexandrides and Smith (1989) have reported that rat fetal skeletal muscle has two subtypes of ß-subunits with molecular mass of 105 kDa and 95 kDa, whereas adult skeletal muscle has only the 95 kDa subtype. These two ß-subtypes reportedly yielded two peptides upon deglycosylation with the same difference in molecular mass, suggesting that the two subtypes may be generated through different post-translational processing. It has also been reported that there are two

types of IGF-I receptors in neuroblastoma cells probably resulting from differential glycosylation (Ota et al., 1988).

The Type I receptor has high-affinity for IGF-I and low-affinity for insulin (Massage and Czech, 1982). The mitogenic action of insulin observed at supraphysiological concentrations in many in vitro experiments is believed to be mediated via the Type I IGF receptor (Rechler and Nissley, 1985). It is also known, however, that insulin can stimulate mitogenesis by binding to its own receptors in hepatoma and teratocarcinoma cells (Massague et al., 1982; Nagarajan and Anderson, 1982). Relative to IGF-I, IGF-II usually exhibits several-fold lower affinity for the Type I IGF receptor and mitogenic activity commensurate to its binding affinity, but in some instances, both IGFs are comparable in their binding affinities for the Type I IGF receptor (Nissley and Rechler, 1984; Rechler and Nissley, 1985). Casella et al. (1986) have reported that human placental Type I IGF receptors purified by immuno-affinity chromatography using a monoclonal antibody αIR-3 specific for the Type I IGF receptor α-subunit (Kull et al., 1983), had similar affinities for both IGF-I and -II. The αIR-3 antibody, however, preferentially inhibited the binding of IGF-I to the purified receptor, which was interpreted to suggest that the Type I IGF receptor had two binding sites only one of which was blocked by the αIR-3. Perdue et al. (1991) also observed similar binding affinities of the Type I IGF receptor from human term placenta for rIGF-I, hIGF-II and a 15 kDa glycosylated hIGF-II variant bearing 21 amino acids of the E-domain, but their competitive binding results fit a 1-binding site model better than a 2-site model. It is thus unclear at present if the Type I IGF receptor has an IGF-II-

specific high-affinity binding site. In any event, the mitogenic action of IGF-II is mediated via Type I IGF receptors in most cases (Van Wyk et al., 1985). Conover et al. (1986) have shown that IGF-II-stimulated DNA synthesis in fibroblasts was blocked by αIR-3. In L6 myoblasts, the insulin-like effect, as well as the mitogenic activity of IGF-II, appears to be mediated via Type I IGF receptors. Kiess et al. (1987) reported that the stimulatory effect of IGF-II on glucose and amino acid transport was less than that for IGF-I and was not blocked by a monoclonal antibody specific to the Type II IGF receptor.

After a ligand binds to the Type I IGF receptor, the two ß-subunits of the disulfide-linked receptor complex are trans-phosphorylated by each other at one or more tyrosine residues (Jacobs et al., 1983; Rubin et al., 1983; Boni-Shinetzler et al., 1988). The intracellular signal of the activated receptor kinase appears to be propagated via serine kinases (Czech et al., 1988; Czech, 1989), although biochemical linkages between the activated receptor and serine kinases and subsequent biological responses are not known. Receptor aggregation has also been implicated in the signaling pathway (Ikari et al., 1988) based on an observation that αIR-3 plus anti-mouse IgG stimulated glycogen synthesis in HEP G2 cells to the same extent as did IGF-I. In contrast to this, Steel-Perkins et al. (1988) reported that the αIR-3 mimicked the biological actions of IGF-I even in the absence of anti-mouse IgG and without causing the activation of tyrosine phosphorylation of the Type I receptor ß-subunit and its putative substrates. The biochemical bases for these phenomena have not been explained.

Insulin and Type I IGF receptors may share signal transmission pathways (receptor cross-talk). Rubin et al. (1983) reported that insulin receptors were phosphorylated by the Type I IGF receptor kinase. Similarly, it was reported that insulin caused phosphorylation of the Type I IGF receptor in L6 myoblasts at concentrations of insulin at which it would have preferentially bound to its own receptor (Beguinot et al., 1988). There is also evidence suggesting that insulin and IGF-I receptors can form disulfide-linked heteroreceptors in cells where both receptors are abundant (Czech, 1989). The existence of the heteroreceptor complex and the cross-phosphorylation of receptors may explain why similar cellular responses are elicited by insulin and IGF-I. However, the physiological significance of such receptor cross-talk remains to be understood.

Type II IGF (IGF-II) Receptors

The Type II IGF receptor is functionally and structurally distinct from insulin and insulin-related receptors. Unlike many other growth factor receptors, the Type II IGF receptor lacks intrinsic tyrosine kinase activity (Czech, 1989; Ullrich and Schlessinger, 1990). The Type II IGF receptor is a single-chain glycoprotein with an apparent molecular mass of 250 kDa including 20-30 kDa of carbohydrate moieties. This receptor has high-affinity for IGF-II, much lower or no affinity for IGF-I, and no affinity for insulin (Massague and Czech, 1982; Sara and Hall, 1990). The apparent size of this protein is ~50 kDa smaller than is predicted from the nucleotide sequence of the receptor cDNA, which may be due to inaccuracy of molecular mass estimation by SDS-PAGE (Morgan et al., 1987). Human IGF-II receptors have a predicted molecular mass of 270 kDa (2451

amino acids), comprising 15 Cys-rich repeats of ~150 residues each, a 23-residue putative transmembrane domain and a short (164 residues) COOH-terminal cytoplasmic domain (Morgan et al., 1987). Both human and rat IGF-II receptors have a ~80% amino acid sequence homology with the bovine cation-independent mannose 6-phosphate receptor (CIM6P) (Morgan et al., 1987; Lobel et al., 1987; MacDonald et al., 1988). The cDNA cloning for hCIM6P revealed that the IGF-II receptor and CIM6P are one and the same. This receptor has two binding sites for IGF-II and CIM6P (Oshima et al., 1988; MacDonald et al., 1988). Mannose 6-phosphate is a marker for lysosomal enzymes. It is therefore an enigma why a receptor protein serves such seemingly unrelated functions, namely as a growth factor receptor and a scavenger of lysosomal enzymes that have escaped intracellular trafficking to the lysosome. Mannose 6-phosphate is known to enhance the binding of IGF-II to the IGF-II/CIM6P receptor (MacDonald et al., 1988). However, IGF-II and a M6P-bearing protein are mutually inhibitory to each other's binding, which may result from steric hindrance or allosteric conformational changes induced by the occupancy of one binding site (MacDonald, 1991).

A 240 kDa truncated form of the Type II IGF receptor lacking COOH-terminal amino acids circulates in the blood (MacDonald et al., 1989). This form of Type II IGF receptor has been shown to be released from a variety of rat tissues <u>in vitro</u> (Bobek et a., 1991) and to be 3-4 times more abundant in fetal and neonatal rat serum than in adult serum (MacDonald et al., 1989), which is reflective of overall abundance of the intact Type II receptors in fetal versus postnatal tissues (Sklar et al., 1989). These results indicate that serum Type II IGF receptors originate

in multiple tissues. Physiological roles for this truncated form of the Type II IGF receptor remain unknown.

The signal transmission pathway for the Type II receptor has been partially characterized. Nishimoto et al. (1987) have shown that IGF-II binding stimulated calcium influx and DNA synthesis via a pertussis toxin-sensitive G protein in EGF-primed BALB/c 3T3 cells, implicating calcium influx as a mitogenic signal. Subsequently, Okamoto and Nishimoto (1991) found that the Type II receptor directly interacted with $G_{i\text{-}2\alpha}$ and that $G_{\beta\gamma}$ potentiated the stimulation-G_{α} coupling in a stoichiometric manner for G_{α}.

Biological responses mediated via the IGF-II/CIM6P receptor are not well defined. In fact, there are two opposing opinions as to the role of this receptor. One view presented by Nishimoto et al. (1987) is that this protein is a mitogenically active receptor. In this regard, IGF-II at low concentrations has been reported to stimulate mitogenesis via the IGF-II receptor in human breast cancer cells, although it elicited its effects via the IGF-I receptor when the growth factor was present at high concentrations (Mathieu et al., 1990). Sklar et al. (1989) found high expression of this receptor in rat fetal tissues compared to tissues from postnatal rats and suggested a role for this protein in fetal growth and development. Scott et al. (1990) found elevated expression of this protein in regenerating rat liver which led the authors to suggest a role for the IGF-II/CIM6P in cell proliferation and/or tissue remodeling. In contrast, other researchers suggested a role for this protein in a major degradative pathway for IGF-II. Kiess et al. (1987) reported that the Type II IGF receptor did not mediate IGF-II stimulation of glucose and amino acid transport in L6 myoblasts, but that blocking the Type II

receptor resulted in an inhibition of IGF-II degradation in culture media by greater than 90%. Also, the enhancing effect of insulin on Type II receptor recycling in isolated rat adipocytes (Oka et al., 1984, 1987; Oka and Czech, 1986) has been implicated as a hypothetical mechanism whereby high levels of insulin might decrease IGF-II actions by increasing the receptor-mediated IGF-II internalization and subsequent degradation, as well as by lowering the IGF-II binding to the Type I receptor (Czech, 1989).

Tissue Expression of IGF Receptors

Type I IGF receptors are expressed at levels comparable to those of insulin receptors only in skeletal muscle and bone tissues. This explains the preferential effect of IGFs on skeletal growth (Czech, 1989). Type I receptor ligands down-regulate these receptors (Rechler and Nissley, 1985), presumably through ligand-induced receptor internalization. The order of the potency of ligands on Type I IGF receptor down-regulation is IGF-I $\geq$ IGF-II $>>$ insulin which is also the order of the binding affinity.

IGF receptors, like the IGFs, are expressed ubiquitously. In general, the Type I receptor mRNA or protein is more abundant in fetal tissues than in adult tissues (D'Ercole et al., 1976; Alexandrides et al., 1989; Werner et al., 1989; Gruppsuo et al., 1991). It is noteworthy that the postnatal decline in Type I receptor expression in the rat is consistent with the developmental pattern of tissue IGF-I expression with the exception of liver (Lund et al., 1986). In the rat uterus, the Type I IGF receptor is expressed mainly in the myometrial layer and its biosynthesis may be under the control of estrogen (Ghahary and Murphy, 1989). In contrast, in cycling, early pregnant and pseudopregnant gilts,

Type I IGF receptors were almost evenly distributed in the myometrium and endometrium regardless of pregnancy or gonadal steroid status (Hofig et al., 1991b). As stated earlier, the Type I IGF receptor in the intestinal mucosa is expressed at high levels in newborn pigs and this is correlated temporally with the rapid intestinal tissue growth and development (Schober et al., 1990).

Type II IGF receptor mRNA and protein levels in tissues also decline after birth in rats. This temporal decline is more precipitous than that of the Type I receptor abundance in this species (Sklar et al., 1989; Alexandrides et al., 1989; Ballesteros et al., 1990). This temporal trend is similar to the developmental expression of IGF-II mRNAs in this species. A narrow (~2-fold) temporal variation in Type II receptor binding capacity and affinity in fetal lamb liver during the latter half of pregnancy has been described (Owens et al., 1985), but the implication of this finding is not yet clear.

Type I IGF receptors have been detected in mammary tissues of several species including the pig (Gregor and Burleigh, 1985; Pekonen et al., DeHoff et al., 1988; Lavandero et al., 1990). In rats, mammary Type I receptor levels peaked at day 5 of pregnancy and then declined continuously during the remainder of pregnancy and lactation (Lavandero et al., 1990). The decline in levels of this receptor during the latter half of pregnancy was also observed in bovine mammary tissues (Hadsell et al., 1990). However, in this species, the receptor levels increased during early lactation (DeHoff et al., 1988; Hadsell et al., 1990), which was interpreted to suggest that IGF-I may play an important role in modulating the metabolic activity of the lactating mammary gland (Hadsell et al., 1990).

The molecular mechanisms underlying regulation of IGF receptor biosynthesis, trafficking and affinity in mammary and other tissues are poorly understood. Estrogen has been reported to increase Type I IGF receptor numbers and mRNA levels in human MCF-7 breast cancer cells, whereas progestin, androgen and dexamethasone were without effect (Stewart et al., 1990). It is not known, however, if these steroids have similar effects in normal mammary tissue.

Type II IGF receptors were also detected in rat, human and bovine mammary tissues (Barenton et al., 1987; Pekonen et al., 1988; Hadsell et al., 1990). In contrast to the levels of Type I receptors, the Type II receptor levels in bovine mammary tissues did not exhibit any temporal variation during pregnancy and lactation (Hadsell et al., 1990). Data concerning the temporally regulated expression of the Type II receptor for the mammary glands of other species are not available.

CHAPTER 3

ONTOGENY OF INSULIN-LIKE GROWTH FACTORS (IGF-I AND IGF-II) AND IGF-BINDING PROTEINS IN PORCINE SERUM DURING FETAL AND POSTNATAL DEVELOPMENT

Introduction

The insulin-like growth factors are peptides evolutionarily conserved from fish to mammals (Dawe et al., 1988; Daughaday and Rotwein, 1989; Sara and Hall, 1990; Humbel, 1990; Rotwein, 1991). Porcine IGF-I is identical in amino acid sequence to human IGF-I (Tavakkol et al., 1988; Francis et al., 1989), and except for one amino acid substitution, porcine IGF-II is identical to its human counterpart (Francis et al., 1989). Both IGFs are found in plasma as well as a variety of biological fluids and are known to reach the target tissue in an endocrine as well as paracrine/autocrine manner (Daughaday and Rotwein, 1989; Van Wyk and Lund, 1989; Sara and Hall, 1990; Humbel, 1990). Developmental changes in circulating concentrations of IGFs have been characterized in humans, rats and sheep (Daughaday and Rotwein, 1989). It is of interest that IGF concentrations change most prominently during the perinatal period. Generally, IGF-I levels are low during fetal life and increase after birth suggesting that the endocrine mode of IGF-I action has increased significance after birth (Daughaday and Rotwein, 1989). On the other hand, IGF-II levels are high during fetal life and decrease immediately after birth in rats and sheep (Moses et al., 1980; Mesiano et al., 1989), whereas in humans, IGF-II

levels increase after birth (Zapf et al., 1981). It is unclear why, in spite of the evolutionary conservation of this peptide, developmental patterns of serum IGF-II levels are widely variable among mammalian species. Pre- and post-natal changes in serum IGF-I levels of pigs have been reported separately (McCusker et al., 1988; Simmen et al., 1988b). Developmental changes in serum IGF-II of pigs have, however, not been elucidated.

The IGF peptides are invariably associated with IGF-binding proteins in biological fluids (Baxter and Martin, 1989a). When the IGF-binding activity of human serum is analyzed by Western ligand blotting, five IGFBPs with apparent molecular mass of 41.5 kDa, 38.5 kDa, 34 kDa, 30 kDa, and 24 kDa are detected (Hardouin et al., 1987). Similar IGFBPs have been reported for rat and porcine sera (Yang et al., 1989; McCusker et al., 1989). It is generally agreed that the ~40 kDa doublet and the 34 kDa IGFBPs represent IGFBP-3 glycosylation variants and IGFBP-2, respectively (Zapf et al., 1988; Hardouin et al., 1989; Veomett et al., 1989). The 30 kDa IGFBP likely represents a mix of IGFBPs (Yang et al., 1989; Hardouin et al., 1989). The identity of the 24 kDa IGFBP is uncertain. Developmental changes in IGFBP levels in pigs have been elucidated only for the prenatal period (McCusker et al., 1989). Therefore, the purpose of this study was to characterize the expression of serum IGFs and IGFBPs throughout fetal and postnatal development in the pig.

Materials and Methods

Blood samples

Sexually-mature crossbred gilts from the Swine Research Unit of the University of Florida were mated and assigned randomly to days 60, 75, 90, 105, and 112 (term = d 114) of gestation. On each of the assigned days, two gilts were hysterectomized while under anaesthesia induced with thiamylal sodium (Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO) and maintained with halothane (Fort Dodge Laboratories, Inc., Fort Dodge, IA) administered via a closed-circuit gas anaesthetic machine. Three fetuses were randomly selected from each gilt and blood was taken from the umbilical vein at the time of surgery. Maternal blood was taken from the radial vein during the surgical period. Neonatal piglets were obtained from seven litters. On days 2, 5, 10, 15, and 21 postnatal, piglets were anaesthetized with halothane gas and blood was obtained by heart puncture. The piglets were then sacrificed with thiamylal sodium and saturated KCl to obtain tissues for a related study. Blood samples from 6-wk-old piglets and prepubertal pigs (164-174 days of age) were obtained at the University of Florida Swine Research Unit and at slaughter, respectively. Blood samples were allowed to clot at room temperature for 1-2 h before preparation of serum. Serum was stored in microfuge tubes at -20 C until analyzed.

IGF-I and IGF-II radioimmunoassay

Recombinant human IGF-I (^{59}Thr) and IGF-II were obtained from Amgen Biologicals (Thousand Oaks, CA). One microgram of each peptide was iodinated to a specific activity of 150-350 μCi/μg protein using 1 mCi Na^{125}I (Amersham, Arlington Heights, IL) and 2.5 μg Iodogen (Pierce,

Rockford, IL). Iodinated IGFs were purified on a Sephadex G-50 (fine) column, and aliquots were stored at -20 C until used.

Serum IGF-binding proteins (IGFBPs) were removed using Sep-Pak^R plus C_{18} cartridges (Waters, Milford, MA) following the procedure of Dr. E. Martin Spencer (personal communication; University of California, San Francisco, CA). Briefly, 0.2 ml serum was acidified with 1.3 ml 1% aqueous trifluoroacetic acid (TFA) for 10 min at 24 C. The acidified serum was loaded on the cartridge which had been pre-conditioned by sequential washes of 100% acetonitrile, deionized water and 0.1% aqueous TFA. After flushing the cartridge with three 1 ml volumes of 0.1% aqueous TFA to remove IGF-binding proteins, the retentate containing free IGFs was eluted in 2 ml acetonitrile containing 0.1% TFA. The acetonitrile eluate was air-dried in a 37 C water bath, and solubilized in 4 ml RIA buffer [30 mM phosphate, pH 7.5; 0.02% protamine sulfate; 10 mM EDTA; 0.05% Tween-20; 0.02% sodium azide]. The suspension was centrifuged for 30 min at 3,000 rpm, and a 10 or 20 μl aliquot (0.5 or 1 μl native serum equivalent) was used for RIA. The assay aliquot was incubated with IGF-I antiserum [UBK 487 (distributed by the National Hormone and Pituitary Program); final dilution of 1:20,000] and 15,000-20,000 cpm [^{125}I]IGF-I in RIA buffer (total volume, 0.5 ml) for 16-18 h at 4 C. Then 0.1 ml of 1:10 sheep antiserum to rabbit gamma globulin was added and the mixture incubated 1 h followed by addition of 0.1 ml 1:100 normal rabbit serum and incubation for an additional 1 h at 4 C. After addition of 1 ml RIA buffer, the tubes were centrifuged for 30 min at 3,000 rpm at 4 C. The supernatant was aspirated and the pellet counted. The IGF-II RIA was performed using the same suspension of Sep Pak-separated serum (10 μl, 0.5 μl native serum

equivalent), 0.2 ng mouse monoclonal IgG against rat IGF-II (Amano Co., Troy, VA) and 15,000-20,000 cpm [^{125}I]IGF-II in the same RIA buffer (0.5 ml total volume) under the same incubation conditions as above. The antigen-antibody complexes were precipitated as for IGF-I RIA using goat antiserum to mouse IgG and normal mouse serum (Sigma Chemical Co., St. Louis, MO). To determine specificity of the IGF-II antibody, crossreactivities with human IGF-I, mouse epidermal growth factor and bovine insulin (the latter two proteins from Collaborative Research Inc., Bedford, MA) in the IGF-II RIA were determined. In each RIA, recombinant human IGF-I and IGF-II were used as reference standards, respectively, and the IGF content was determined from logit-log plots. The maximum binding of [^{125}I]IGF was 40-50% of total counts with less than 2% nonspecific binding in each RIA. A significant displacement of radiolabeled ligand and half-maximum binding occurred at 5 pg (5-8% displacement) and 55-65 pg unlabelled IGF-I in the IGF-I RIA and 20 pg (7-13% displacement) and 90-110 pg unlabelled IGF-II in the IGF-II RIA, respectively.

IGF-binding activity

To assess the effectiveness of the Sep-Pak procedure in removing serum IGF-binding proteins, IGF-binding activity was measured before and after Sep-Pak chromatography, following the procedure of Baxter et al. (1983) with minor modifications. In brief, after reconstitution of air-dried Sep-Pak eluates with RIA buffer, graded doses of the eluates and raw serum were incubated with 50,000 cpm [^{125}I]IGF-II for 2 h at 24 C in a total volume of 0.4 ml. The incubation mixture received an equal volume of ice-cold 1% charcoal suspension in RIA buffer and was incubated for 8 min at 24 C. After centrifugation for 30 min at 3,000 rpm, 0.4 ml

supernatant was removed and counted. To further confirm the effectiveness of the procedure, an acetonitrile eluate of acidified serum obtained by Sep-Pak chromatography was incubated with [^{125}I]IGF-II and subjected to Sephadex G-100 gel exclusion chromatography, followed by measurement of radioactivity in fractions as detailed in the legend to Figure 3-1.

Western ligand blotting

SDS-PAGE was performed according to the procedure of Laemmli (1970). Two or two and one-half microliters of serum were mixed with 23 μl distilled water and 25 μl 2X sample buffer [1X: 62.5 mM Tris, pH 6.8; 5% SDS; 10% sucrose; 0.02% bromophenol blue]. After boiling for 3 min, the mixture was loaded onto a 3% stacking gel, and electrophoresed through a 12.5% polyacrylamide slab gel at 25 mA/gel under nonreducing conditions. Protein mol wt standards (Bio-Rad, Richmond, CA) were electrophoresed in adjacent lanes. After electrophoresis, proteins were transferred onto nitrocellulose membranes (0.2 μm, Schleicher and Schuell, Inc., Keene, NH) overnight at 200 mA at 4 C in Towbin buffer [25 mM Tris, pH 8.3; 192 mM glycine; 20% methanol] using a Hoefer (San Francisco, CA) electrophoresis transfer unit. Ligand blotting was performed following the procedure of Hossenlopp et al. (1986) with minor modifications. After the electrotransfer, the nitrocellulose membranes were air-dried, washed with TBS [10 mM Tris, pH 7.4; 150 mM NaCl and 0.05% sodium azide], and incubated with 1% non-fat dry milk in TBS on a rocking platform for 1 h at 24 C. The membranes were washed with TBS containing 0.1% Tween-20 for 10 min and then incubated with [^{125}I]IGF-I or [^{125}I]IGF-II (0.6 x 10^6 cpm/100 cm^2 membrane) in TBS containing 0.1% Tween-20 and 1% BSA (Sigma) in plastic bags for 20-24 h at 4 C on a rocking platform. After rinsing the

membranes with TBS, they were washed twice with TBS containing 0.1% Tween-20 and twice with TBS on a rocking platform for 15 min per wash. Membranes were air-dried, and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY) at -80 C for 7-14 days. Autoradiograms were scanned using an LKB densitometer (Pharmacia, Piscataway, NJ).

Deglycosylation

Deglycosylation of serum proteins was performed using N-glycanase (Genzyme Corp., Boston, MA) according to the procedure of Yang et al. (1989) with minor changes. Briefly, 2 μl serum was incubated with 3 U N-glycanase at 37 C for 24 h in 30 μl (total volume) of 0.3 M sodium phosphate buffer, pH 8.6, containing 1.25% Triton X-100, 0.17% SDS, and 5.75 μM PMSF. After incubation, the reaction mixture was mixed with an equal volume of 2X SDS-gel sample buffer, boiled for 3 min, and subjected to the Western ligand blotting procedure.

Immunoprecipitation

Ten microliters of porcine serum were incubated with 5 μl rabbit antiserum to porcine IGFBP-3 (Walton et al., 1989) or rat IGFBP-2 and 85 μl 50 mM sodium phosphate, pH 7.4, overnight at 4 C. The mixture then was added to 150 μl washed protein A-Sepharose beads (Pharmacia, Piscataway, NJ) along with 500 μl 50 mM Tris, pH 7.4, and the mixture incubated overnight at 4 C on a rotating shelf. After washing the insoluble Protein A-Sepharose beads three times with Tris buffer containing 0.5% Triton X-100, the pellet was mixed with 150 μl 2X SDS-gel sample buffer, boiled for 5 min, centrifuged, and a 60 μl supernatant (2 μl native serum equivalent) was subjected to Western ligand blotting. The specificity of the IGFBP-3 antiserum has been reported previously (Walton and Etherton, 1989). The

IGFBP-2 rabbit antiserum (gift from Dr. K. T. Shiverick, University of Florida) was raised against rIGFBP-2 purified from conditioned media of astrocytes cultured from 21-day-old rats (Olson et al., 1991). The NH_2-terminal sequence of the purified rIGFBP-2 was identical to that of BRL-3A IGFBP (rIGFBP-2) (Mottola et al., 1986).

Statistical analysis

Analysis of variance of IGFs-I and -II concentrations was performed using the procedure of General Linear Model of SAS (1986). The analysis included correlation between and regression on age of these variables.

Results

IGF-I and IGF-II radioimmunoassays

Serum IGF-binding proteins were almost totally removed by acidification and chromatography on a Sep-Pak cartridge. Most of the serum IGF-binding activity was found in the Sep-Pak pass-through and 0.1% TFA eluate with a negligible proportion present in the acetonitrile eluate (Figure 3-1A). Consistent with this result, the acetonitrile eluate incubated with [^{125}I]IGF-II exhibited no radioactive peak corresponding to an [^{125}I]IGF-II-IGFBP complex during G-100 gel exclusion chromatography, while raw serum exhibited two peaks of [^{125}I]IGF-II-IGFBP complexes at 150 kDa and 40 kDa molecular mass regions (Figure 3-1B). Further, the acetonitrile eluate was parallel to recombinant hIGF-I and hIGF-II in dose-response displacements of [^{125}I]IGF-I (Figure 3-2, top) and [^{125}I]IGF-II (Figure 3-2, lower). Human IGF-I has 10% crossreactivity in the IGF-II RIA according to specifications accompanying the IGF-II antibody and this was confirmed in the present study (Figure 3-2, lower). Although not

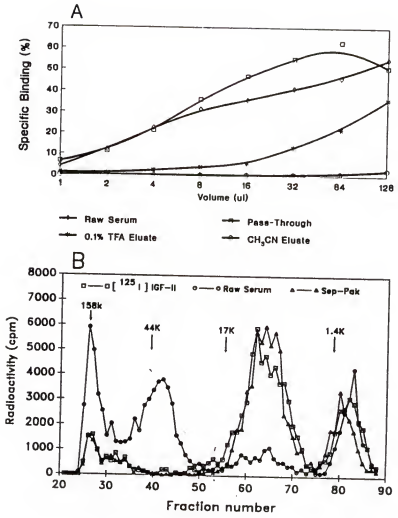

Figure 3-1. IGF-binding activity of Sep-Pak eluates of acidified porcine serum. A. IGF-binding activity of Sep-Pak eluates of acidified porcine serum and raw serum measured by the charcoal method. Six hundred microliters of a composite serum was acidified with 1% TFA, loaded on two Sep-Pak cartridges, flushed with 0.1% TFA, and eluted with acetonitrile containing 0.1% TFA as described in Materials and Methods. After air-drying and reconstitution of each eluate with 0.6 ml RIA buffer, IGF-binding activity of the eluate and of raw serum was measured by a charcoal method using 50,000 cpm of purified [^{125}I]IGF-II. The [^{125}I]IGF-II was 89.4% precipitable by the charcoal procedure. B. G-100 gel exclusion (2.5 X 49 cm) chromatography of raw serum and acetonitrile eluate of acidified serum after incubation with unpurified [^{125}I]IGF-II. Acetonitrile eluate (0.4 ml native serum) from a Sep-Pak cartridge or 0.4 ml of raw serum was incubated with 100,000 cpm [^{125}I]IGF-II in elution buffer (0.05 M phosphate, pH 7.5) for 2 h at 24 C in a total volume of 2 ml, eluted through the G-100 column, and 3 ml fractions were collected. The radioactivity peaks near the void volume and total volume for [^{125}I]IGF-II correspond to aggregated [^{125}I]IGF-II and free ^{125}I, respectively.

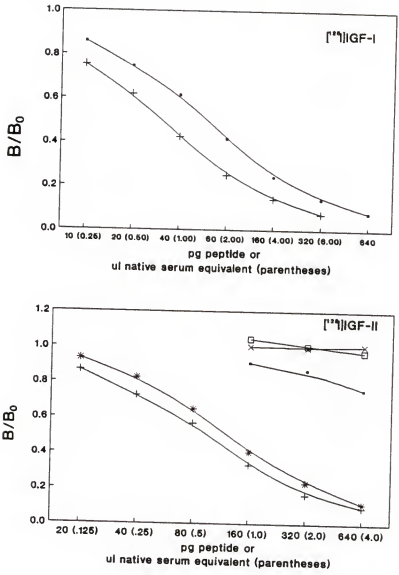

Figure 3-2. Dose-response displacements of [^{125}I]IGF-I (top) and [^{125}I]IGF-II (lower) from its corresponding antibody by recombinant hIGF-I (■), hIGF-II (*), porcine serum stripped of IGF-binding proteins by Sep-Pak chromatography (+), bovine insulin (□), and mouse epidermal growth factor (x). The serum was a pool of sera form several 10-day-old neonatal piglets. B/Bo represents the ratio of the counts at each dose (B) divided by the counts at zero dose (Bo) after subtracting the nonspecific counts.

shown in the figure, IGF-II, insulin and EGF had negligible cross-reactivity (< 1%) in the IGF-I RIA. The separation efficiency of the Sep-Pak cartridge procedure was 75-80% as estimated from recovery of radiolabeled IGFs added to serum 2 h prior to separation. There were no differences in separation efficiency for IGF-I and IGF-II. Thirteen percent of [^{125}I]IGF added to serum was lost during the loading step and an additional 3% was lost during subsequent flushing of the Sep-Pak cartridge with 0.1% trifluoroacetic acid. When unlabelled IGF-I or IGF-II standard was added to serum, 72.2% of added IGF-I and 73.8% of added IGF-II were measured in the IGF-I and IGF-II RIAs, respectively, indicating that over 90% of added IGF was measured by RIA when corrected for column separation efficiency. Within- and between-assay coefficients of variation were 4.9% and 8.1% in the IGF-I RIA and 5.5% and 7.2% in the IGF-II RIA, respectively.

Serum concentrations of IGF-I and IGF-II

Serum concentrations of IGF-I in pig fetuses were low and exhibited little change up to day 90 of gestation (Figure 3-3, top). A two-fold increase in serum IGF-I was evident during the last three weeks before term (day 114). Concentrations of IGF-I in serum of newborn piglets continued to increase and at 3 wk of age exceeded that for maternal serum (Figure 3-3, top). In contrast, concentrations of IGF-II remained high throughout the prenatal period, and tended to decrease slightly near term (Figure 3-3, lower). The IGF-II concentrations then increased postnatally, but the change in IGF-II levels was not as pronounced as that for IGF-I. There was a significant correlation between serum IGF-I and IGF-II concentrations up to day 42 postnatal ($r^2 = 0.656$, $P = 0.0001$).

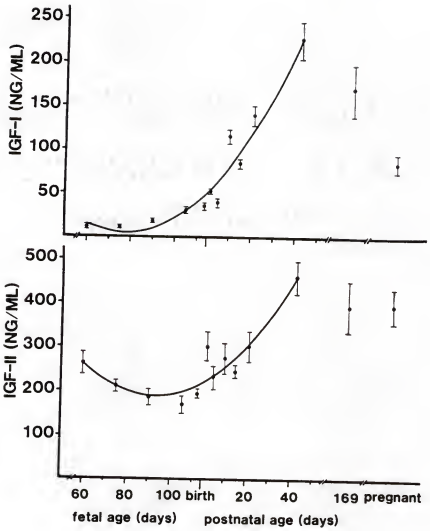

Figure 3-3. IGF-I (top) and IGF-II (lower) concentrations in porcine serum. The IGF-II concentrations were corrected for the cross-reactivity of IGF-I (10%) in the IGF-II RIA. Each point and bar represents the mean ± SEM of serum IGF concentrations of six fetuses or five to seven postnatal pigs. IGF-I and IGF-II concentrations up to day 42 postnatal fit the quadratic regression on age (day): $y1 = 16.37 - 1.348x + 0.0372 x^2$ ($r^2 = 0.798$, $P = 0.0001$) and $y2 = 262.89 - 4.533x + 0.0686x^2$ ($r^2 = 0.575$, $P = 0.0001$), where y1 and y2 represent IGF-I and IGF-II concentrations (ng/ml) and x = days after conception - 60, respectively.

Western ligand blotting

Serum IGF-binding activity was characterized by Western ligand blotting using [^{125}I]IGF-I or [^{125}I]IGF-II. Seven IGFBP bands with apparent molecular mass of 43 kDa, 39 kDa, 34 kDa, 31 kDa triplets, and 26 kDa were detected in cycling gilts (Figure 3-4A). In subsequent analysis of sera from fetal and postnatal pigs, the 31 kDa triplet appeared as a diffuse and broad band (Figure 3-4B). Although the autoradiographic intensities of the IGFBP bands appeared to be diminished by switching the ligand from [^{125}I]IGF-I (Figure 3-4A) to [^{125}I]IGF-II (Figure 3-4B), the IGFBP signals for fetal and postnatal sera were in fact intensified by [^{125}I]IGF-II with no obvious change in relative intensity of individual IGFBP bands when the two ligands were compared in several experiments (data not shown). The differences in relative intensities of the 43 kDa, 39 kDa and 34 kDa bands among prepubertal (day 169), cycling and pregnant (day 105) gilts (Figure 3-4A,B) suggest a change in distribution of serum IGFBPs during this period. To quantify the relative abundance of each IGFBP, the autoradiogram (Figure 3-4B) for fetal and postnatal sera was scanned by laser densitometry (Table 3-1).

The abundance of the 43 kDa and 39 kDa IGFBPs increased during late fetal and early postnatal development. This roughly paralleled the increase in concentrations of IGF-I (Figure 3-3). Interestingly, the abundance of the 43 kDa and 39 kDa IGFBPs increased 4-fold between day 112 fetal and day 2 postnatal stages, whereas levels of the 34 kDa IGFBP decreased 2-fold during the same period. The 31 kDa IGFBP was detectable only in sera from postnatal piglets and, together with the 26 kDa band, represented a minor portion of the total IGF-binding activity in porcine

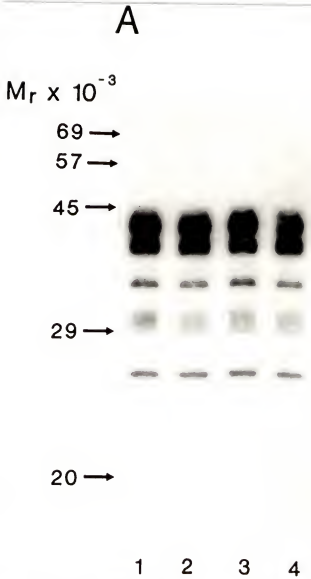

Figure 3-4. Western ligand blotting of IGF-binding proteins in porcine serum. A. Two and one-half microliters of sera from cycling gilts were separated on a 12.5% polyacrylamide gel (lanes 1-4), electroblotted onto a nitrocellulose membrane, probed with [^{125}I]IGF-I, and visualized by autoradiography. B. Two microliters of composite serum from fetal and postnatal pigs (lanes 1-7) and day 105 pregnant gilts (lane 8) were subjected to the ligand blotting as in (A) except that [^{125}I]IGF-II was used. The estimated molecular mass of the IGFBPs are 43 kDa, 39 kDa, 34 kDa, 31 kDa, and 26 kDa. C. Composite maternal (M) and fetal (F) sera were subjected to deglycosylation by N-glycanase (lanes 3, 4) or immunoprecipitation by rabbit antisera to pIGFBP-3 (lanes 5, 6), rIGFBP-2 (lanes 7,8), and normal rabbit serum (NRS) (lanes 9, 10) before western ligand blotting. Lanes 1 and 2 are for untreated sera. In each lane, 2 μl native serum or its equivalent was separated and probed with [^{125}I]IGF-II.

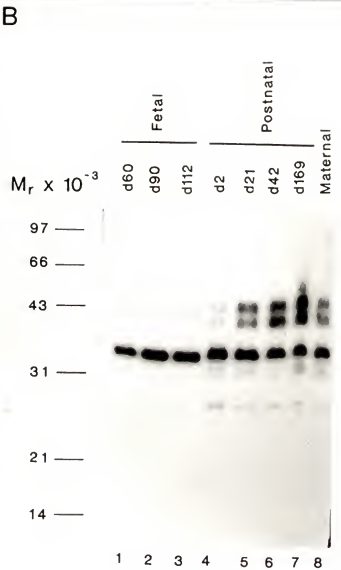

Figure 3-4--continued

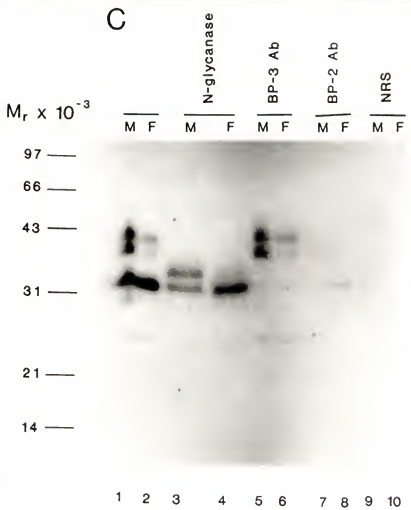

Figure 3-4--continued

Table 3-1. Relative Autoradiographic Intensities of IGFBPs Monitored by Western Ligand Blotting[a]

	Fetal (days)			Postnatal (days)				
Size (kDa)	60	90	112	2	21	42	169	Gilt
43	11	12	10	26	99	102	101	21
39	1	2	4	33	56	68	45	44
34	309	437	401	171	190	128	86	46
31	UN[b]	UN	UN	30	36	27	21	12
26	20	14	9	26	23	26	21	22

[a]The intensities of IGFBP bands in Figure 3-4B were quantitated by scanning densitometry. The intensity of the 39 kDa IGFBP band of day 60 fetal serum was arbitrarily assigned a unit density.
[b]UN, undetectable.

sera. These latter two proteins exhibited no obvious developmental changes in abundance.

To characterize the immunologic relatedness of the observed IGFBPs with known IGFBPs, fetal and maternal sera were subjected to immunoprecipitation with rabbit antisera to pIGFBP-3 and rIGFBP-2. Immunoprecipitates were then analyzed by ligand blotting. The IGFBP-3 immunoprecipitate was characterized by the 43 kDa and 39 kDa bands and by minor bands at 31 kDa and 26 kDa evident upon long autoradiographic exposure (30 days) (Figure 3-4C). Ligand blotting of IGFBP-2 immunoprecipitated from fetal serum revealed the 34 kDa band, which was less apparent in the immunoprecipitate from maternal serum. As a rule, the relative autoradiographic intensity for each IGFBP band in immunoprecipitates paralleled that from crude serum.

To identify IGFBPs with N-linked glycosylation, fetal and maternal sera were treated with N-glycanase prior to electrophoresis and ligand blotting. The 43 kDa and 39 kDa IGFBP doublet was reduced to a single 36.5 kDa band following deglycosylation, whereas the 34 kDa IGFBP was unaffected (Figure 3-4C). The 31 kDa and 26 kDa IGFBP bands were undetectable after deglycosylation, presumably due to their low abundance and recovery.

Discussion

Insulin-like growth factors-I and -II are partially GH-dependent peptides thought to be important mediators of fetal and postnatal growth and development (Daughaday and Rotwein, 1989; Sara and Hall, 1990; Humbel, 1990). Both hIGF-I and rIGF-II, when infused through the renal artery,

have been reported to increase the growth and differentiation of fetal rat paws transplanted under syngeneic kidney capsules (Liu et al., 1989). The growth-promoting effects of IGF-I have been documented in hypophysectomized (Schoenle et al., 1985) as well as normal (Hizuka et al., 1985) rats. Although IGF-II was thought initially to be primarily a fetal growth factor, there is evidence to suggest IGF-II is also an important postnatal growth factor. Like IGF-I, IGF-II has a variety of metabolic and growth-promoting actions in vitro that are mediated through IGF-I (Type I) and IGF-II (Type II) receptors (Sara and Hall, 1990; Humbel, 1990; Rechler and Nissley, 1985). Further, circulating concentrations of IGF-II are higher than those of IGF-I during postnatal life in humans, sheep, and cattle (Zapf et al., 1981; Mesiano et al., 1989; Badinga et al., 1991). In the work of Shaar et al. (1989) and van Buul-Offers et al. (1988), IGF-II increased weight gain and other growth indices in hypophysectomized rats and Snell dwarf mice, respectively.

Developmental changes in IGF gene expression, mRNA levels and protein synthesis and secretion have been best characterized in rats (Lund et al., 1986; Daughaday and Rotwein, 1989; Hall and Sara, 1990). In this species, IGF-I gene expression in nonhepatic tissues is high during fetal development and low postnatally, whereas hepatic IGF-I gene expression increases postnatally accompanied by markedly increased serum IGF-I concentrations (Lund et al., 1986; Donovan et al., 1989). IGF-II gene expression, on the other hand, parallels serum IGF-II concentrations in both hepatic and nonhepatic tissues with the exception of brain IGF-II mRNA expression (Lund et al., 1986). Thus, in the rat, IGF-I may assume,

in part, the role of IGF-II after birth and at the same time, IGF-I action may switch from paracrine/autocrine to endocrine modes.

The birth-related increase in serum IGF-I levels observed here may reflect maturation of the somatotropic axis. Serum IGF-I concentrations are highly correlated with hepatic GH receptor binding in rats and domestic animals (Gluckman et al., 1983b; Maes et al., 1984; Breier et al., 1989; Bandinga et al., 1991). That the somatotropic axis is functional to some extent during late fetal ontogeny is suggested by the finding of decreased serum IGF-I levels following fetal hypophysectomy in pigs and sheep (Mesiano et al., 1989; Jewell et al., 1989). Thus, it is likely that GH regulates, in part, IGF-I production beginning at late fetal stages and perhaps coincident with GH receptor ontogeny.

The porcine IGF-II profile is somewhat novel in that no abrupt change in concentrations occurred during the late fetal or perinatal periods as was observed for some other species (Daughaday and Rotwein, 1989). The preponderance of IGF-II over IGF-I in fetal serum and the postnatal increases in serum levels of both IGFs support the view that IGF-II is a fetal and postnatal growth factor, whereas IGF-I may be primarily a postnatal growth factor in pigs. The idea that the increased availability of both IGF peptides during early postnatal development plays a significant role in perinatal growth regulation is buttressed by the demonstrated presence of Type I IGF receptors in neonatal pig tissues (Schober et al., 1990) and by the known growth-promoting actions of both IGFs in postnatal rodents (Schoenle et al., 1985; Hizuka et al., 1985; van Buul-Offers et al., 1988; Philipps et al., 1988; Shaar et al., 1989).

IGFBPs have several postulated functions. The IGFBPs potentially serve as a plasma reservoir for IGFs by increasing their plasma half-lives (Zapf et al., 1986). In this regard, plasma IGF concentrations are one to two orders of magnitude higher than is necessary to attain maximal biological responses in vitro (Nissley and Rechler, 1984; Florini, 1987). IGFBPs may protect the host from the acute insulin-like effects of free IGFs (Zapf et al., 1986). In addition, IGFBPs serve to transport IGFs across the vascular endothelium (Bar et al., 1990) as well as modulate the biologic actions of IGFs at the cell surface (Clemmons et al., 1986).

In addition to IGFBPs -1, -2 and -3, there are at least three other IGFBP species identified to date. Shimonaka et al. (1989) isolated a 36/32 kDa (reducing) IGFBP doublet from adult rat serum which was homologous to an IGFBP isolated from culture media of human TE89 osteosarcoma cells (Mohan et al., 1989). This IGFBP reportedly is the second most abundant in adult rat serum (Shimonaka et al., 1989). Zapf et al. (1990) recently isolated a different IGFBP from adult human serum with an apparent molecular mass of 28-30 kDa which has an NH_2-terminal amino acid sequence homologous to the 32-34 kDa IGFBP isolated from human cerebrospinal fluid (Roghani et al., 1989) and conditioned medium of human AG 2804 fibroblast (Martin et al., 1990) and human He[39]L lung fibroblast (Forbes et al., 1990) cell cultures.

The present Western ligand blotting results were generally similar to data reported previously for humans and rats (Hardouin et al., 1987; Yang et al., 1989; Donovan et al., 1989). The immunologic relatedness of the 40 kDa doublet (43, 39 kDa) to pIGFBP-3 and the conversion of this doublet to a single band of 36.5 kDa by N-glycanase treatment, consistent with

known characteristics of IGFBP-3 (Zapf et al., 1988; Hardouin et al., 1989; Yang et al., 1989; Coleman and Etherton, 1991), identifies the 40 kDa IGFBPs as IGFBP-3 glycosylation variants. The 31 kDa and 26 kDa IGFBP bands detected in pIGFBP-3 immunoprecipitates may represent truncated forms of IGFBP-3, as purified serum pIGFBP-3 exhibits minor bands at ~30 kDa and ~25 kDa in SDS-PAGE (Walton et al., 1989). The coordinate developmental increases in abundance of pIGFBP-3 and IGF-I is likely to be GH-driven since serum levels of these peptides are dependent on GH status (Buonomo et al., 1988; Daughaday and Rotwein, 1989; Hardouin et al., 1989; Yang et al., 1989; Sara and Hall, 1990; Humbel, 1990). Alternatively, a GH→IGF-I→IGFBP-3 cascade may be functional since IGF-I stimulates secretion of IGFBP-3 in hypophysectomized rats (Zapf et al., 1989) and human fibroblast cultures (Hill et al., 1989).

The immunologic relatedness of the 34 kDa IGFBP to rIGFBP-2 and its apparent lack of carbohydrate moieties identify this protein as porcine IGFBP-2. The transition from fetus to neonate was characterized by a marked decrease and increase in abundance of the 34 kDa and 40 kDa IGFBPs, respectively. In view of the inverse relationship of GH status and serum levels of the 34 kDa IGFBP (Hardouin et al., 1989; Coleman and Etherton, 1991), it is plausible that the somatotropic axis matures early in pigs.

The broad 31 kDa band is resolved into a triplet of IGFBPs in ligand blotted porcine serum. One of the triplet bands likely represents a C-terminal truncated form of IGFBP-3 as purified pIGFBP-3 and rIGFBP-3, having only one NH_2-terminal sequence, exhibited a band at ~30 kDa in addition to higher molecular mass doublets (Zapf et al., 1988; Walton et al., 1989). If either or both of the 36/32 kDa IGFBP and the 28-30 kDa

IGFBPs that were recently isolated from rat (Shimonaka et al., 1989) and human (Zapf et al., 1990) sera, respectively, also circulate in porcine serum, the 31 kDa IGFBP band may also include these new IGFBP(s). It is also uncertain whether IGFBP-1 is a component of the 31 kDa IGFBP band. Although McCusker et al. (McCusker et al., 1990, 1991) detected a ~30 kDa IGFBP by immunoblotting porcine fetal and neonatal sera using antiserum specific for hIGFBP-1, Mondschein et al. (1990, 1991) were unable to detect the porcine homologue of IGFBP-1 in conditioned media of porcine granulosa cell cultures or in porcine follicular fluid and serum. The 26 kDa IGFBP appears in pIGFBP-3 immunoprecipitates, although its exact identity remains unclear. In terms of their relative contributions to serum IGF transport as estimated from abundance, the smaller IGFBPs are minor compared with the 40 kDa and 34 kDa IGFBPs.

CHAPTER 4

EXPRESSION OF mRNAs ENCODING IGFs, IGFBPs, AND IGF RECEPTORS IN PORCINE TISSUES DURING FETAL AND POSTNATAL DEVELOPMENT

Introduction

It is generally believed that the liver is the primary source of plasma IGFs (Daughaday and Rotwein, 1989; Sara and Hall, 1990). By virtue of the large size of this organ and its central role in synthesis of major serum proteins, hepatic output of IGF-I in the primary hepatocyte culture (Scott et al., 1985a) or liver perfusion systems (Schwander et al., 1983) can account for the bulk of circulating IGF-I pool in the rat. The correlation between plasma IGF levels and hepatic IGF mRNA levels during development also supports these findings (Donovan et al., 1989; Adamo et al., 1989). However, in nonrodent species, similar studies have not been performed and the developmental patterns of IGF-I expression in hepatic and nonhepatic tissues are unknown. To examine if liver is the major site of IGF production and to further assess the dynamics of IGF production and uptake in the pig, the expression levels of IGFs, IGF receptors and IGF-binding proteins in tissues and serum during fetal and postnatal development of pigs were measured.

Materials and Methods

Tissue and serum preparation

Fetal pigs were obtained at hysterectomy (days 60 and 90) and Caesarian section (day 112) of gilts under general anesthesia as described in the previous chapter. Piglets at days 10, 21, and 42 postnatal were obtained from one litter. Brain, lungs, liver, kidneys, and hindlimb muscles were removed from the piglets after euthanization with thiamylal sodium and saturated KCl, immediately frozen in liquid nitrogen and stored at -80 C until analyzed. Blood samples were obtained from the umbilical vein for days 60 and 90 fetuses and by heart puncture for all other pigs, and serum was prepared and stored at -20 C until used. Blood and liver samples from adult (~170 days old) pigs were obtained after slaughter.

RNA isolation

Individual tissue samples for each day were pooled (3-7 samples/pool) and pulverized at -20 C to minimize degradation of RNA. Total cellular RNA was isolated by the guanidine thiocyanate method as modified by Puissant and Houdebine (1990). RNA preparations from liver and muscle were additionally treated with lithium chloride to remove polysaccharides (Puissant and Houdebine, 1990). Polyadenylated (poly(A^+)) RNA was enriched from total RNA by oligo(dT) cellulose-chromatography (Maniatis at al., 1982). Final RNA concentrations were estimated from optical absorbance at 260 nm (absorbance unit = 33 μg RNA/ml).

Preparation of cDNA probes

A porcine IGF-I cDNA clone sigf.3 was prepared as described by Tavakkol et al. (1988). Other cDNA clones were used for transformation of Escherichia coli by the $CaCl_2$ method and the plasmid DNAs were isolated

using the CsCl centrifugation method (Maniatis et al., 1982). The cDNA inserts were purified using Geneclean II (Bio 101, La Jolla, CA) after restriction-endonuclease digestion and agarose gel electrophoresis. A pGH receptor cDNA clone pMON 2268, containing a 2.9 kb insert, was provided by G. G. Krivi (Monsanto Company, Saint Louis, MO). This cDNA clone was digested with EcoRI and XhoI and the resulting mixture of 1.1 and 1.8 kb cDNA fragments was used for nick-translation. A 780-base pair IGF-II cDNA subcloned in pUC 12 (Whitfield et al., 1984) was provided by M. M. Rechler (National Institutes of Health, Bethesda, MD). This fragment encodes amino acids 38-156 of pre-pro-IGF-II and also contains 3' untranslated sequences. A rat IGF-binding protein (IGFBP)-2 cDNA, subcloned in pGem 3 (plasmid A1; Brown et al., 1989), was also provided by M. M. Rechler. A fragment containing IGFBP-2 coding sequences as well as lambda phage DNA sequences was isolated after HindIII digestion. A human type I IGF receptor cDNA clone pIGF-I-R.8 (Ullrich et al., 1986) in pUC 13 harboring a 730-base pair EcoRI-insert was from American Type Culture Collection, Rockville, MD. Plasmid p119, containing a 3 kb EcoRI-fragment encoding the rat type II IGF receptor (MacDonald et al., 1988) was provided by M. P. Czech (University of Massachusetts, Worcester, MA). This plasmid DNA was digested with EcoRI and XmnI and the resulting mixture of 3 kb cDNA and the two vector fragments were used for nick-translation. The human IGFBP-3 cDNA clone pibp118.1 (Wood et al., 1988) containing a nearly full-length cDNA fragment was a gift from W. I. Wood (Genentech, South San Francisco, CA). The human rRNA gene clone pA4 in pBR322 containing a 7 kb insert including part of the 18S gene, both internal transcribed spacers, the 5.8S gene, and nearly full-length 28S gene (Erickson et al., 1981;

Gonzalez et al., 1985) was provided by R. D. Schmickel (University of Pennsylvania, Philadelphia, PA). A 770-base pair chicken ß-actin cDNA fragment was purchased from Oncor (Gaithersburg, MD).

Northern hybridization

Five micrograms of poly(A^+)-enriched RNA were separated in a 1.5% agarose gel containing 2.2 M formaldehyde following the procedure of Thomas (1980). After electrophoresis, RNA was transferred to a nylon membrane (Biotrans, ICN, Irvine, CA) and baked for 2 h at 80 C. cDNA probes with a specific activity of ~1 X 10^8 cpm/µg were prepared using a nick-translation kit (Amersham). After prehybridization for 2 h at 42 C in prehybridization solution containing 2X SSC (1X = 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 50% formamide, 5X Denhardt's solution (1X = 0.02%, w/v, for each of Ficoll, polyvinylpyrrolidone and BSA), 40 mM sodium phosphate, pH 6.5, 0.1% SDS, and yeast RNA (250 µg/ml), the membranes were hybridized with 1-5 X 10^6 cpm cDNA/ml in fresh prehybridization solution containing 1X Denhardt's solution overnight at 42 C. The filters were washed with 2X SSC-0.1% SDS at room temperature for 30-60 min followed by 0.1X SSC-0.1% SDS for 15-60 min at 60 C, unless otherwise indicated and exposed to X-Omat RP films (Eastman Kodak, Rochester, NY) with intensifying screens at -80 C. Sizes of mRNAs were calculated on the assumption that lengths of 28S and 18S rRNAs, as visualized by ethidium bromide staining, are 5 kb and 2 kb, respectively.

IGF-I and IGF-II RIAs

Recombinant hIGF-I and hIGF-II were obtained from Amgen Biologicals (Thousand Oaks, CA). Both peptides were iodinated using Chloramine-T to a specific activity of ~300 µCi/µg as described previously (Lee and

Henricks, 1990). Serum IGFBPs were removed by acidification and C_{18} Sep-Pak chromatography prior to IGF RIA as described in chapter 3. For the measurement of tissue IGF concentration, tissues were extracted with acetic acid and the extract subjected to C_{18} Sep-Pak chromatography. In brief, 1 gm of tissue was homogenized in 19 ml of 1 M acetic acid and incubated on ice for 2 h (D'Ercole et al., 1984). A 1.5 ml aliquot was centrifuged at 12,000 x g for 10 min and 1 ml of the supernatant loaded on a C_{18} Sep-Pak cartridge which had been preconditioned by sequential 3 ml washes of CH_3CN, water, and 1 M acetic acid. After washing the cartridge with 5 ml of 1 M acetic acid, the retentate was eluted in 3 ml of CH_3CN containing 0.1% trifluoroacetic acid. The eluate was evaporated to dryness and reconstituted in 4 ml of RIA buffer (0.03 M sodium phosphate, pH 7.5, 0.02% (w/v) protamine sulfate, 0.01 M EDTA, 0.05% (v/v) Tween-20, and 0.02% (w/v) sodium azide), and 128 μl (1.6 mg native tissue equivalent) used in RIA for each IGF. The recovery after Sep-Pak chromatography of ^{125}I-IGF added to the tissue homogenate was 62%, with no difference between IGF-I and IGF-II. IGF measurements were adjusted for column recovery as well as for contributions from residual blood. This latter adjustment was done after measuring hemoglobin concentration (Crosby and Furth, 1956) in the tissue homogenate using a purified porcine hemoglobin preparation (Sigma) as standard. Percentage of serum of residual blood in the tissue was calculated on the assumptions that the hematocrit and hemoglobin concentration in blood increased from 27% to 40% and from 85 mg/ml to 130 mg/ml, respectively, during day 60, fetal through day 170, postnatal (Pond and Maner, 1984). IGFs-I and -II RIAs were performed as described in chapter 3.

Affinity-Crosslinking of ^{125}I-IGF-I to Type I IGF Receptors

Microsomal membranes were prepared as described by Hofig et al. (1991b). To remove the endogenous IGF ligands from their receptors, the membrane pellet was reconstituted in tissue homogenization buffer (Hofig et al., 1991b) containing 3 M $MgCl_2$ (final concentration), incubated for 30 min at 4 C, centrifuged at 100,000 X g for 80 min, and the final pellet was reconstituted in membrane storage buffer (Hofig et al., 1991b) and stored at -80 C until used. Membrane protein concentrations were determined by the method of Lowry et al. (1951) using BSA as standard. Procedures used for the affinity-crosslinking of ^{125}I-IGF-I to type I IGF receptors were as described by Hofig et al.(1991b).

Western Ligand Blotting of Membrane Type II IGF Receptors and Serum IGFBPs

The procedure used for identification of membrane Type II IGF receptors was described by Mathieu et al. (1990). Briefly, 200 μg membrane proteins were solubilized in 0.33X RIPA lysis buffer (Burr et al., 1980) (1X = 0.1 M sodium phosphate, pH 7.2, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, and 200 kallikrein inhibitor units of aprotinin/ml) for 1 h at 4 C with shaking. After removal of insoluble material by a brief centrifugation, an aliquot corresponding to 150 μg original membrane protein was mixed with SDS-PAGE sample buffer, boiled for 3 min and subjected to 7.5% SDS-PAGE under nonreducing conditions. Separated proteins were electro-transferred to a nitrocellulose membrane. The membrane was immersed in a modified quenching buffer (10 mM Tris, 0.9% NaCl, 0.1% Tween-20, 0.3% Triton X-100, 0.2% BSA, 1% nonfat dry milk, and 0.1% NaN_3) for 24 h at 4 C and then incubated with ^{125}I-IGF-II (80,000

cpm/ml) in binding buffer (25 mM Tris, pH 7.4, and 10 mM $MgCl_2$) for 3 h at room temperature. The membrane was washed (2 x 15 min) with Tris buffer, pH 7.4, containing 10 mM $MgCl_2$ and 0.1% Tween-20 and then with 20 mM Tris containing 10 mM $MgCl_2$ for 20 min. Western ligand blotting of serum IGFBPs was performed as described in chapter 3.

Results

Expression of mRNAs Encoding GH Receptors

Northern blot hybridization of poly(A^+)-enriched RNA preparations from porcine tissues with a pGH receptor cDNA identified a GH receptor mRNA with a size of 4.6 kb (Figure 4-1, top left) which was resolved into a doublet upon extended electrophoresis (Figure 4-2, top right). Poly(A^+)-enriched RNA preparations from brain and day 42 postnatal kidney were judged to contain impurities which was revealed from low intensities of EtBr-stained RNAs of these preparations after electrophoresis; hence, these were excluded from the Northern analyses. The GH receptor mRNAs were expressed at higher levels in muscle and kidney than in liver and lung during the perinatal period, although temporal changes in levels of GH receptor mRNAs were different among tissues. A developmental increase in the level of GH receptor mRNAs was apparent in the liver, with the exception of a peak at day 112 of fetal life, but a reverse trend was apparent for the muscle. In the lung and kidney, levels of GH receptor mRNAs peaked at day 90 of fetal development and tended to increase with postnatal development.

Consistency of RNA loading and transfer was confirmed by repeated Northern hybridization experiments as well as by rehybridization of

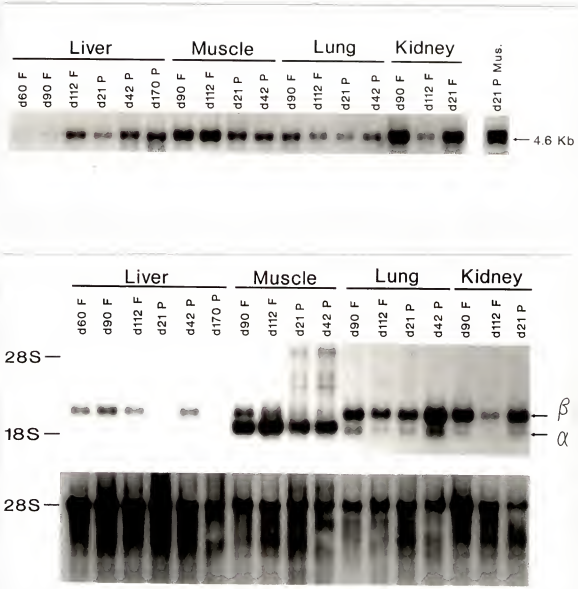

Figure 4-1. Developmental expression of GH receptor mRNA. Upper, 5 μg poly(A$^+$)-enriched RNA were subjected to Northern blot analysis with a pGH receptor cDNA probe as described in "Materials and Methods." Middle, the GH receptor Northern filter was rehybridized without prior stripping to a chicken ß-actin cDNA probe (6 x 10^2 cpm/ml). Bottom, the ß-actin cDNA-probed filter was rehybridized with a human 28S ribosomal DNA probe (7 x 10^5 cpm/ml). Autoradiographic exposure times were (upper) 72 h, (middle) 48 h, and (lower) 8 h.

Northern filters with different probes. Rehybridization of the GH receptor Northern filter with a chicken ß-actin cDNA fragment revealed 2.2 kb ß-actin as well as 1.5 kb α-actin mRNA (Figure 4-2, middle). Levels of the ß-actin mRNA were somewhat variable among tissue types and developmental stages, which did not resemble results of any other Northern blots presented here. A second rehybridization of the ß-actin-probed Northern filter with a human 28S ribosomal DNA probe revealed a slightly higher level of contamination of 28S rRNA in poly(A^+)-enriched RNA preparations from liver as compared to other tissues (Figure 4-2, bottom). Nonetheless, this difference was not considered significant in the data interpretation.

Expression of mRNAs Encoding IGF-I and IGF-II

IGF-I mRNAs with sizes of 7.8 and 1.0 kb were detected by Northern hybridization of poly(A^+)-enriched RNAs preparations from liver, muscle, lung and kidney (Figure 4-2, upper left). The levels of IGF-I mRNAs in the liver were relatively low compared with those in nonhepatic tissues and did not increase during postnatal development. A second Northern hybridization experiment performed with newly prepared RNAs and a reduced stringency wash protocol revealed 6.6, 5.2 and 3.9 kb IGF-I mRNAs in addition to the 7.8 and 1.0 kb mRNAs in the muscle and liver (Figure 4-2, upper right). The 1.0 kb mRNA was more abundant in day 170 postnatal liver than in day 21 liver or muscle, yet the order of abundance of the 7.8 kb mRNA for the three tissues was the same as in the first Northern blot. The level of IGF-I mRNAs in muscle was highest at day 90 fetal followed by day 21 postnatal. The transient increase in the IGF-I mRNA levels at day 90 fetal was also apparent in lung and kidney.

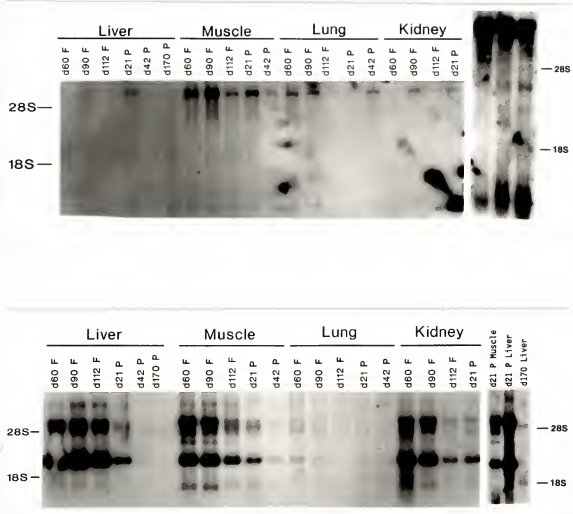

Figure 4-2. Developmental expression of mRNAs encoding IGF-I and IGF-II. Upper and lower left, 5 μg poly(A$^+$)-enriched RNA were subjected to Northern blot analysis with (upper) a pIGF-I cDNA probe and (lower) a rat IGF-II cDNA probe, respectively and washed at 60 C as described in "Materials and Methods." Upper and lower right, poly(A$^+$)-enriched RNAs (5 μg) from day 21 postnatal (left lane) muscle and (middle lane) liver and (right lane) day 170 liver were hybridized as for left and washed at 50 C. Autoradiographic exposure times were (upper left) 10 days, (upper right) 7 days, (lower left) 5 days, and (lower right) 32 h.

Multiple IGF-II mRNAs with sizes of 9.1, 6.3, 5.8, 4.7, 3.8, 2.9, and 1.7 kb were detected after Northern blot hybridization with a rat IGF-II cDNA (Figure 4-2, lower left). Of the observed IGF-II mRNAs, the 6.0 kb doublet (6.3 and 5.8 kb) and 2.9 kb mRNAs predominated in all tissues. The levels of IGF-II mRNAs were highest in the liver followed by muscle, kidney, and lung. There were, however, developmental and tissue-specific aspects of IGF-II mRNA expression. Levels of the IGF-II mRNAs were high at days 60 and 90 fetal and declined markedly during the peri-natal period in all tissues examined. The 4.7 and 3.8 kb IGF-II mRNAs were detected only in muscle and kidney. When Northern hybridization was performed with a reduced stringency wash protocol, low levels of 5.5 and 1.9 kb IGF-II mRNAs were detected in the day 170 postnatal pig liver (Figure 4-2, lower right).

Concentrations of IGF-I and IGF-II in Tissues and Sera

IGF-I concentrations in sera were low during fetal development and increased postnatally. Serum IGF-II concentrations, which exceeded IGF-I concentrations by an order of magnitude in fetuses, declined transiently at day 90 fetal life, but exhibited a gradual increase thereafter. The acetic acid extracts of reference tissues after chromatography on C_{18} Sep-Pak columns were parallel to the unlabeled IGF standards in displacing ^{125}I-IGFs from their corresponding antibodies (Figure 4-3). The Sep-Pak chromatography step was used, since with increasing doses, the starting acetic acid extract was less competitive than free IGF in displacing ^{125}I-IGF, presumably due to incomplete removal of IGFBPs by acetic acid extraction alone (data not shown). This lack of parallelism was more apparent in the IGF-II RIA. IGF-I concentrations for liver were low

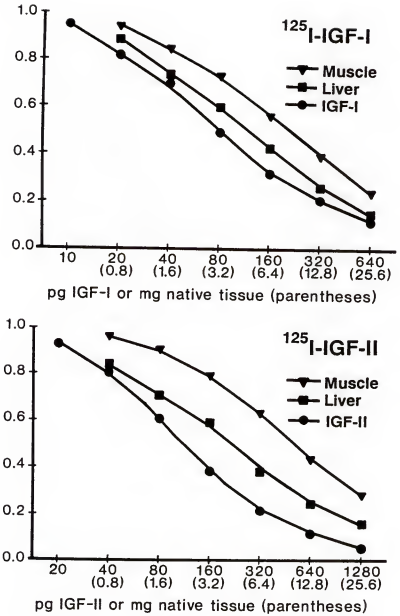

Figure 4-3. Dose-response displacements of ^{125}I-IGF-I and ^{125}I-IGF-II from their corresponding antibodies by tissue extracts. Muscle and liver were extracted with 1 M acetic acid and the extract was chromatographed on C_{18} Sep-Pak columns as described in "Materials and Methods." The acetonitrile eluate from the C_{18} column was evaporated and reconstituted with RIA buffer. Upper, displacements of ^{125}I-IGF-I from its antibodies by recombinant hIGF-I standard and tissue extracts. Lower, displacements of ^{125}I-IGF-II by recombinant hIGF-II and tissue extracts.

Table 4-1. Concentrations of IGF-I and IGF-II in Tissues and Sera of Fetuses and Postnatal Pigs[a]

	Fetal			Postnatal		
	day 60	day 90	day 112	day 21	day 42	day 170
IGF-I (ng/ml or gm tissue)						
Serum	11.5±3.6	10.1±1.6	14.6±9.3	90.6±9.3	91.4±15.8	182.3±30.1
Brain	10.2	15.0	ND[b]	ND	ND	ND
Lung	8.5	7.7	2.6	4.6	25.6	ND
Liver	0.8	3.4	4.0	9.2	12.8	0.6
Kidney	ND	18.4	10.1	33.2	ND	ND
Muscle	20.6	20.8	3.4	9.3	11.0	ND
IGF-II (ng/ml or gm tissue)						
Serum	185.3±10.5	87.5±6.0	126.3±6.9	220.3±7.6	232.1±24.4	326.0±19.1
Brain	94.1	55.1	ND	ND	ND	ND
Lung	178.0	114.0	64.5	61.0	77.3	ND
Liver	217.5	88.3	103.6	99.9	83.2	62.8
Kidney	ND	324.1	176.3	181.6	ND	ND
Muscle	296.9	172.7	70.0	81.0	44.5	ND

[a]IGF concentrations for sera represent means ± SEM of three or four observations. IGF concentrations for tissues are single observations. Tissues from three or four pigs were pooled and the pooled tissues extracted with 1 M acetic acid followed by C_{18} Sep-Pak chromatography (Materials and Methods). The tissue IGF concentration was corrected for a contribution from residual blood on the assumption that this was uniform among individual tissues within a pool.

[b]ND, not determined.

in fetuses and transiently increased in neonatal pigs. IGF-I concentrations were high in fetal muscle than in muscle of postnatal pigs, whereas in lung, IGF-I concentration was greatest at day 42 postnatal. IGF-II concentrations were, in general, greater in fetal tissues than in postnatal tissues. Regardless of this generalization, kidney exhibited relatively high concentrations of both IGFs.

Types I and II IGF Receptors

Three distinct mRNAs with estimated sizes of 11, 7, and 6 kb were detected by Northern blot hybridization with a human type I IGF receptor cDNA fragment (Figure 4-4, upper). The 11 kb mRNA was the most abundant of these mRNAs in all RNA preparations, but the relative abundance of each size of mRNA was variable among tissues. The temporal alterations in Type I IGF receptor mRNA expression were variable among the tissues. In the liver, levels of these mRNAs were low during the latter half of fetal life and after a transient decrease and an increase at days 21 and 42 postnatal, respectively, declined to almost undetectable levels at day 170. The receptor mRNA levels in muscle did not change during the fetal period, but declined during neonatal development. In lung and kidney, in contrast, mRNA levels were high at days 60 and 90 fetal, transiently declined by day 112, and increased after birth. Temporal expression levels of type I IGF receptor mRNAs in these two organs paralleled the abundance of IGF-I mRNAs.

Affinity-crosslinking of $MgCl_2$-treated microsomal membrane proteins with ^{125}I-IGF-I followed by SDS-PAGE identified a 135 kDa band corresponding to a complex of the α-subunit of the Type I IGF receptor and its ligand (Figure 4-4, lower). This receptor exhibited the highest

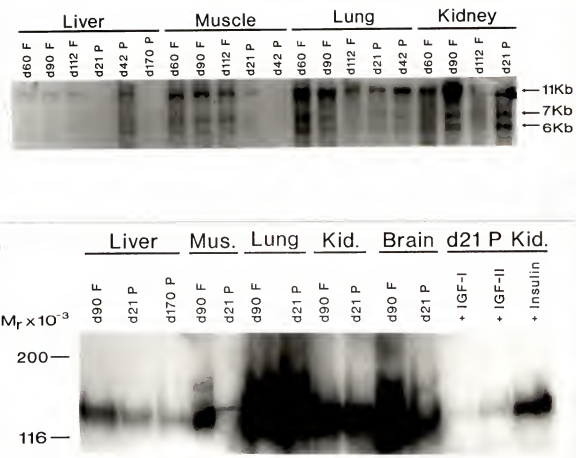

Figure 4-4. Developmental expression of the Type I IGF receptor and its mRNAs. Upper, Poly(A^+)-enriched RNA (5 μg) was hybridized with a human type I IGF receptor cDNA probe as described in "Materials and Methods." Autoradiographic exposure time was 10 days. Lower, $MgCl_2$ (3 M)-treated membrane proteins (1 mg) were incubated with 1 x 10^5 cpm ^{125}I-IGF-I in 1 ml assay buffer (10 mM Tris, pH 7.8, containing 0.5% BSA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3) for 24 h at 4 C (right three lanes) with or without 0.2 μg unlabeled peptides. The membranes were centrifuged for 10 min at 12,000 x g and the pellet was reconstituted with 0.5 ml of 60 mM HEPES, pH 7.5, and crosslinked with disuccinimidyl suberate (0.1 mM) for 30 min at 4 C. After terminating the crosslinking reaction with 1 ml TE (10 mM Tris, pH 7.8, 1 mM EDTA), membrane proteins were pelleted (12,000 x g for 10 min), mixed with SDS-PAGE sample buffer, boiled for 5 min, and electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions (5% 2-mercaptoethanol). The gel was dried and exposed to a slow film with an intensifying screen for 6 days at -80 C.

relative affinity for IGF-I followed by IGF-II and insulin as evident by the competitive displacements of ^{125}I-IGF-I by excess unlabeled peptides (Figure 4-4, lower). The abundance of the Type I IGF receptor proteins as indicated by the autoradiographic intensity of the 135 kDa band was high for fetal and postnatal lung and kidney and for fetal brain compared to liver and muscle. In day 90 fetuses, the Type I IGF receptor abundance was highest for brain and lung followed by kidney, muscle and liver. Levels of this receptor declined after birth in brain, muscle and liver, whereas in lung and kidney, the high receptor abundance persisted after birth. The lack of the 135 kDa band for the day 21 postnatal muscle was due to a loss of proteins during the procedure. In a second experiment, a low level of the Type I IGF receptor was detected for a comparable sample for which the $MgCl_2$ treatment step was omitted (data not shown).

A 9 kb Type II IGF receptor mRNA was detected in all tissues examined (Figure 4-5, upper). In general, the abundance of this mRNA was higher in fetuses than in neonates, although temporal variations in receptor mRNA levels were somewhat tissue-specific. Type II IGF receptor proteins with a molecular mass of 250 kDa were identified by Western ligand blotting of solubilized microsomal membrane preparations (Figure 4-5, lower). The abundance of this receptor as assessed from the autoradiographic intensity of the 250 kDa band was greatest in fetal lung and muscle, followed by kidney, liver and brain. In all tissues examined, the Type II IGF receptor levels were much more lower at day 21 postnatal those at day 90 fetal.

IGFBP-2 mRNA, with a length of 1.5 kb, was detected in the liver and kidney (Figure 4-6, top and bottom left). Although not apparent from the

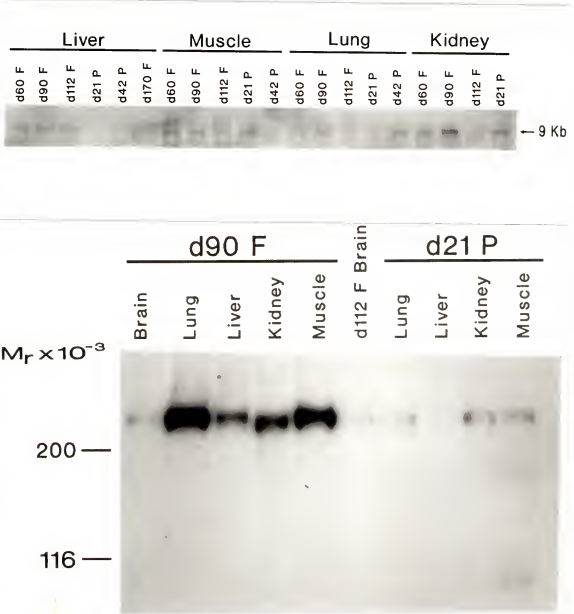

Figure 4-5. Developmental expression of the Type II IGF receptor and its mRNA. Upper, Poly(A$^+$)-enriched RNA (5 μg) was hybridized with a rat Type II IGF receptor cDNA probe as described in "Materials and Methods." The autoradiographic exposure time was 12 days. Lower, RIPA-lysed membrane proteins (150 μg) were subjected to 7.5% SDS-PAGE. Separated proteins were electrically transferred to a nitrocellulose membrane after which the membrane was quenched, incubated with ^{125}I-IGF-II (80,000 cpm/ml) for 3 h at room temperature, washed, dried, and exposed to a fast film with an intensifying screen for 6 days at -80 C as described in "Materials and Methods."

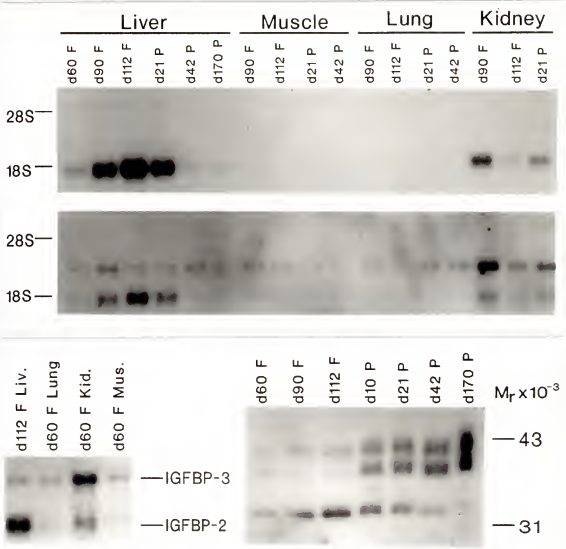

Figure 4-6. Developmental expression of IGFBPs-2 and -3 mRNAs in tissues and IGFBPs in serum. Top and bottom left, poly(A^+)-enriched RNA (5 μg) was hybridized with a rIGFBP-2 cDNA probe as described in "Materials and Methods." Middle and bottom left, the Northern filter used for the IGFBP-2 mRNA study was rehybridized with a hIGFBP-3 cDNA without prior stripping of the previous probe. Autoradiographic exposure times were (for IGFBP-2) 3 days and (for IGFBP-3) 7 days. Bottom right, serum (3 μl) was subjected to 12.5% SDS-PAGE after which separated proteins were electrically transferred to a nitrocellulose membrane. The membrane was blocked with 1% nonfat dry milk in TBS (10 mM Tris, pH 7.4, 0.15 M NaCl, and 0.05% NaN_3) for 1 h at room temperature, washed with TBS-0.1% Tween-20, incubated with ^{125}I-IGF-II (1.2 x 10^5 cpm/ml) for 20 h at 4 C in TBS containing 0.1% Tween-20 and 1% BSA, washed with TBS-0.1% Tween-20 (2 x 15 min) and with TBS (3 x 15 min), dried, and exposed to a slow film with an intensifying screen for 5 days at -80 C. Each sample was a pool of sera obtained from 3-7 animals.

Northern blot, low levels of this mRNA were also found in brain, lung and muscle when analyzed by RNA dot-blot hybridization (data not shown). IGFBP-2 mRNA level was greatest in fetal and neonatal liver. Hepatic IGFBP-2 mRNA abundance increased during the latter half of fetal life and declined to almost undetectable levels by day 42 postnatal. IGFBP-3 mRNA (~3kb) was detected in every tissue examined (Figure 4-6, middle and bottom left). In contrast to the highest abundance of IGFBP-2 mRNA in liver, the IGFBP-3 mRNA was most abundant in the kidney. To compare the variations in IGFBP mRNA expression to changes in circulating levels of IGFBPs, serum levels of IGFBPs were determined by Western ligand blotting (Figure 4-6, bottom right). The abundance of the 34 kDa serum IGFBP, corresponding to IGFBP-2 (Lee et al., 1991a), increased during the latter half of fetal life, and then declined gradually during postnatal development. Levels of the 43 kDa and 40 kDa IGFBPs representing glycosylation variants of IGFBP-3 (Lee et al., 1991a) were low during the fetal period and increased postnatally.

Discussion

IGFs are evolutionarily conserved peptides that are probably expressed in all vertebrates (Rotwein, 1991). The amino acid sequence of pIGF-I is identical to hIGF-I and is different from rIGF-I only in three amino acids (Tavakkol et al., 1988; Rotwein, 1991). pIGF-II is different from hIGF-II and rIGF-II at one and three amino acids, respectively (Rotwein, 1991). Molecular analysis of the human and rat IGF-I chromosomal genes and cloned cDNAs has revealed that by use of alternative promoters, differential splicing and variable sites of polyadenylation,

multiple IGF-I mRNAs of differing lengths are generated (Rotwein, 1991). The multiple IGF-I mRNAs observed in the present study are thus likely to be generated through a similar mechanism(s). However, the preponderance of the 7.8 kb IGF-I mRNA in the porcine fetal and postnatal porcine tissues examined contrasts with the preponderance of the 1.0 kb mRNA in porcine uterus (Tavakkol et al., 1988; Geisert et al., 1991), as well as with the preponderance of the 5.3 and ~1 kb mRNAs in human fetal tissues (Han et al., 1988) and adult liver (Rotwein, 1986) and in adult rat tissues (Murphy et al., 1987).

Multiple IGF-II mRNAs are generated through mechanisms similar to those used for IGF-I mRNA biosynthesis (Schofield, 1991). IGF-II gene expression is regulated in a tissue- and development-specific manner in the human and rat (Scott et al., 1985c), which was also apparent from this study. In the human a 5.5 kb IGF-II mRNA initiated from the P1 promoter is expressed exclusively in adult liver (Scott et al., 1985c; de Pagter-Holthuizen et al., 1988), whereas a 1.8 kb mRNA, which is transcribed from an IGF-II exon and is translated *in vitro* into a protein unrelated to IGF-II, is expressed in both fetal and adult liver (de Pagter-Holthuizen et al., 1988). In this regard, the 5.5 and 1.9 kb mRNAs observed in adult liver may represent porcine homologues of the human 5.3 and 1.8 kb mRNAs.

IGF concentrations in tissues were, in general, reflective of levels of IGF mRNAs and IGF receptor mRNA or protein. The relatively high IGF concentrations for kidney, however, may have reflected the uptake of circulating IGFs for clearance (D'Ercole et al., 1977). These results suggest that tissue IGF concentrations likely represent *de novo*-synthesized as well as receptor-bound and internalized IGF proteins.

Overall, tissue IGF concentrations were similar to previously reported results for porcine tissues (Hausman et al., 1991).

Expression levels in porcine tissues of Type I IGF receptors were also developmentally regulated, with a peri-natal decline in levels of the receptor mRNAs observed. The relative abundance of Type I IGF receptor mRNAs in tissues was consistent with those of membrane-associated Type I IGF receptor proteins as assessed from results of affinity-crosslinking. However, the high level of Type I IGF receptors in day 21 postnatal lung contrasted with a low receptor mRNA level. This disparity may have resulted from a developmental increase in the binding affinity of the receptor in this organ. In this regard, it was previously reported that the binding affinity of IGF-I receptors for porcine fetal lung was greater than those for other fetal organs and that this increased during the later stages of fetal development, whereas such changes in affinity were not apparent for other internal organs (D'Ercole et al., 1976). The peri-natal decline in levels of Type II IGF receptor was more apparent than changes in corresponding mRNA. These differences as well as the discrepancy between the receptor mRNA and protein levels may reflect tissue- and development-specific processing and/or turnover of this receptor (Ballesteros et al., 1990).

The tissue source(s) of circulating IGF-II in adult humans and animals has been somewhat of an enigma, since hepatic IGF-II mRNAs are expressed at very low levels. Traditionally, the liver has been considered the major endocrine source of both IGFs. In line with this concept, Schofield and Tate (1987) postulated that IGF-II may be stabilized by a binding protein in adult serum, or that IGF-II produced

from the adult human liver may be secreted, whereas fetal liver-produced IGF-II is locally utilized without entering the circulation. It is known that the plasma half-life of IGFs is greater when associated with the IGFBP-3-containing 150 kDa IGFBP complex (Baxter and Martin, 1989b) than when associated with smaller classes of IGFBPs including IGFBP-2 (2-4 h vs 8-30 min, respectively; Cohen and Nissley, 1976). This difference in the plasma half-life of IGFs between the two sizes of IGF-IGFBP complexes is apparently related to a difference in the transcapillary movement of the complexes, since low M_r IGFBPs can traverse the endothelium (Bar et al., 1990), whereas the transcapillary movement of the 150 kDa is impeded by endothelium (Binoux and Hossenlopp, 1988). As shown in this study, the transition from IGFBP-2 to IGFBP-3 in porcine serum during the postnatal period is associated temporally with increased circulating levels of IGFs-I and -II. The alternative proposal of these authors (Schofield and Tate, 1987), however, does not have strong supporting evidence, since contradictory to their postulation, both fetal and adult rat liver explants secrete IGFs in culture (Rechler et al., 1979; Binoux et al., 1980). The low IGF-II mRNA and protein levels in adult pig liver, in addition to the reduced mass ratio for the liver per total body weight (15% for the mid-gestation pig fetus vs 1.4% for adult; Pond and Maner, 1984), also renders the alternative hypothesis unlikely. As such, liver is not likely to be the major endocrine source of IGF-II in mature pigs, although this organ is likely to secrete IGF-II into plasma due to its relative scarcity of Type II IGF receptors. It is thus plausible that high circulating levels of plasma IGF-II in adult pigs are due to a decreased plasma clearance rate of IGF-II resulting from both the

postnatal transition of IGFBP-2 to IGFBP-3 in plasma and a decreased tissue uptake of this peptide as a consequence of the postnatal decline in IGF receptor expression.

An unexpected result of this study was the finding that levels of the IGF-I mRNAs in porcine liver were relatively low and unchanged during postnatal development. This is in marked contrast to the high abundance of IGF-I mRNAs in newborn lambs (Dickson et al., 1991) and to a 100-fold increase in the hepatic IGF-I mRNA level which occurs in rats during the first 50 days after birth (Adamo et al., 1989). The postnatal increase in circulating IGF-I levels in the pig may also be due to the developmental transition of IGFBP-2 to IGFBP-3 and to decreased tissue uptake of IGF-I.

The constitutive hepatic expression of IGF-I mRNAs contrasted with the postnatal increase in GH receptor mRNA expression in this organ. The temporal changes in levels of GH receptor mRNAs were similar to those of GH receptor proteins previously reported by Breier et al. (1989). These results indicate that the temporal expression of IGF-I in porcine liver is not correlated with GH receptor levels as was observed for the rat (Maes et al., 1984; Adamo et al., 1989). It remains to be resolved, however, if GH receptor mRNAs detected in hepatic and nonhepatic fetal tissues are translated into functional receptors and if so, whether these receptors regulate tissue expression of IGF-I mRNAs.

In the pig, skeletal muscle may be an important tissue source of circulating IGF-I. Skeletal muscle represents the bulk of total body weight. Secondly, IGF-I (mRNAs and protein) and Type I IGF receptors are expressed at high and low levels, respectively, in hindlimb muscles as shown in this study. Similarly, Semitendinosus muscle and Longissimus

Dorsi muscle have been reported to express IGF-I mRNAs at levels higher than those in livers of growing pigs (Leaman et al., 1990; Grant et al., 1991). These results, however, contrast with those for growing beef cattle where hepatic IGF-I mRNA levels far exceeded those for the Sternomastoid muscle (Hannon et al., 1991). It remains, therefore, to be determined if IGF-I mRNA expression levels are regulated in a muscle-specific fashion in large animals.

It is known that the IGFs mediate tissue growth and development via autocrine, paracrine, and endocrine routes. Based on the present results, both IGFs-I and -II are likely to act preferentially through autocrine/paracrine routes in the fetus, since the IGFs and their receptors are present at high levels in tissues. However, in the fetal lung where IGF-II mRNA levels were low and type II IGF receptor levels high, plasma may be a significant source of IGF-II for this organ. Fetal liver, in contrast, may be a significant endocrine source of IGF-II, as it exhibited the highest level of IGF-II mRNAs and the lowest level of Type II IGF receptors among the tissues examined, with the exception of brain. If this is the case, the transient decline in circulating IGF-II concentrations at day 90 fetal development is attributable to a ~5-fold decline in the proportion of liver to total body weight between days 60 and 90 of fetal development (Widdowson, 1971; Pond and Maner, 1984). In the postnatal period, lung and kidney may constitute major target tissues of plasma IGFs with their high levels of Type I IGF receptors.

As for the IGFs, IGFBPs are thought to be secreted primarily by the liver in the rat (Scott et al., 1985a,b). Results of the present Northern hybridizations, however, do not support this model for larger animals.

Unlike the hepatic predominance of IGFBP-3 mRNA expression in livers of adult rats (Shimasaki et al., 1989), porcine liver did not express IGFBP-3 mRNA at the highest level. Although serum IGFBP-2 levels were correlated with hepatic IGFBP-2 mRNA abundance during the fetal period, the gradual decline of serum IGFBP-2 level during postnatal development contrasted with the rapid postnatal decline of hepatic IGFBP-2 mRNA expression.

In summary, the regulation of expression of IGFs and their receptors and binding proteins in the pig differs substantially from that reported for the rat. In particular, liver does not appear to be the primary source of circulating IGFs in the pig. Since levels of mRNAs encoding IGFs -I and -II did not increase postnatally in any of the porcine tissues examined, the postnatal increases in serum IGF levels in the pig may be due to decreased plasma clearance rates of IGFs, rather than to increased hepatic secretion rates.

CHAPTER 5

EXPRESSION OF THE MAMMARY IGF SYSTEM DURING PREGNANCY AND PSEUDOPREGNANCY: EXAMINATION OF THE ROLES OF THE IGFs AND ESTROGEN IN MAMMARY DEVELOPMENT

Introduction

The majority of mammary gland growth and development takes place during pregnancy in mammalian species (Knight and Peaker, 1982; Tucker, 1987). Although pregnancy-associated mammary development is believed to be elicited by conceptus-derived hormonal factors, as evidenced by reduced mammary growth following removal of conceptuses in the mouse and pig (Nagasawa and Yanai, 1971; Kensinger et al., 1986a), the identities and mechanisms of actions of the putative conceptus mammogens are largely unknown. Placental lactogen may be a conceptus-derived mammogen in some species, but in other species, including the pig, where this protein has not been demonstrated, uncharacterized nonprolactin-like conceptus factors may also promote mammary development (see review by Forsyth, 1986).

Recently, various peptide growth factors including insulin-like growth factors (IGFs)-I and -II have been identified in mammary tissues and in media of cultured mammary epithelial cells (Dickson et al., 1985; Pekonen et al., 1988; Baumrucker and Stemberger, 1989; Winder et al, 1989; Coombes et al., 1990). These growth factors are mitogens for mammary epithelial cells *in vitro* and are therefore potential mammogens (Oka and Yoshimura, 1986; Forsyth, 1989, 1991), with the exception of transforming

growth factor-β which was demonstrated to be an inhibitor of mammary growth *in vivo* in rats (Silberstein and Daniel, 1987). However, molecular mechanisms underlying mammary expression of peptide growth factors and the roles of growth factors in pregnancy-associated growth of mammary glands are largely unknown.

Pseudopregnant gilts have a hormonal status similar to that of pregnant gilts, except for reduced circulating levels of estrogen, and therefore can be used to study the effects of estrogen on pregnancy-associated growth and differentiation of mammary tissues (DeHoff et al., 1986). To gain insights into roles for IGFs and estrogen in mediation of mammary development, the temporally regulated expression of components of the mammary IGF system in pregnant gilts in the presence and absence of conceptuses (pseudopregnancy) and during estradiol administration in pseudopregnant gilts was measured.

Materials and Methods

Animals

Crossbred gilts were mated at the first sign of estrus and were assigned randomly for surgery on days 30, 45, 60, 75, 90, 105, or 112 of pregnancy (three gilts per day; two days per gilt). On the two assigned days, the gilts were anesthetized (Kensinger et al., 1986a) and mammary tissue was removed from the penultimate mammary gland (one gland at each biopsy) using sterile procedures. Mammary tissue was trimmed of fat, immediately frozen in liquid nitrogen and stored at -80 C. A blood sample was taken from a radial vein at the time of surgery. The gestational stage of each pig was confirmed on the basis of known breeding dates and

by examining conceptuses after hysterectomy at the time of the second mammary biopsy. Mammary tissues corresponding to early pregnancy (Px) and lactation were obtained from four day 12 Px gilts and one day 4 postpartum sow, respectively.

Pseudopregnancy was induced by injecting subcutaneously 5 mg estradiol valerate per day on days 11 through 15 of the estrous cycle (Kensinger et al., 1986a). Three pseudopregnant (Ppx) gilts subsequently received a daily injection of 5 mg estradiol valerate from day 45 until the day preceding the second mammary biopsy, whereas another three gilts received vehicle (corn oil) only. Each gilt was biopsied on two randomly assigned days of Ppx, corresponding to days 60, 90 and 112 after onset of estrus. The gilts were hysterectomized at the second mammary biopsy and pseudopregnancy was confirmed by identifying functional corpora lutea and uteroferrin secretion from the purple color of uterine flushings.

Northern Analysis

Mammary tissues were pulverized at -20 C and pooled by day (Px) or day x treatment (Ppx) on an equal weight basis. Total RNA and Poly(A^+) RNAs were isolated following standard procedures (Maniatis et al., 1982; Puissant and Houdebine, 1990). In some instances, poor yields of poly(A^+) RNA necessitated the use of total cellular RNA in the Northern analyses.

All of the cDNA probes used in this study were prepared as described in the previous chapter. The mouse ß-casein cDNA clone pCMß13 (Gupta et al., 1982) (pBR322 + 0.8 kb PstI-insert) and a rat ß-casein cDNA clone 6ßc3' (Richards et al., 1981) (pSP6 + 0.6 kb PstI-insert) were gifts from Dr. Jeffrey M. Rosen (Baylor College of Medicine, Houston, TX). These plasmids were digested with PstI and the resulting mixture of fragments

used for nick-translation. Procedures for Northern blot hybridization were as described in the previous chapter.

Radioimmunoassays for IGF-I and IGF-II

Procedures for iodination and IGF RIAs for sera were described in the previous chapters. For the measurement of IGF content in mammary tissues, the tissues were extracted using acetic acid as in chapter 4 and the acetic acid extracts were subjected to acid-ethanol extraction as described for serum (Daughaday et al., 1980). This was done instead of Sep-Pak chromatography because of blockage of the cartridges by lipids in the extract. After centrifugation at 1,730 x g for 30 min, the acid-ethanol extract was evaporated to dryness and the residue dissolved in IGF RIA buffer. The dose-response displacement curve for the tissue extracts was parallel to that for each rhIGF standard. IGF measurements for the tissues were corrected for the contributions from residual blood as described in the previous chapter, on the assumption that whole blood has a hemoglobin concentration of 130 mg/ml and 40% hematocrit.

Affinity-Crosslinking and Ligand Blotting

Procedures for affinity-crosslinking of membrane Type I IGF receptors and for Western ligand blotting of membrane Type II IGF receptors and of serum IGFBPs were also described in the previous chapter.

Statistical analysis

Analysis of variance of IGF concentrations in sera and tissues was performed using the procedure of General Linear Model of SAS (1986). Day was included in the model for Px gilts. For Ppx gilts, day, treatment (estradiol injection), treatment x day were included in the model.

RESULTS

Mammary GH Receptor mRNAs

Northern blot analysis with a pGH receptor cDNA probe identified 4.5 and 4.7 kb GH receptor mRNAs in all mammary poly (A^+) RNA preparations examined (Figure 5-1, upper). When the Northern filter was rehybridized with a chicken β-actin mRNA to confirm the consistency of loading and transfer, a 2.2 kb β-actin mRNA and a 1.5 kb α-actin mRNA were detected (Figure 5-1, lower), the relative autoradiographic intensities of which were nearly constant across the lanes (Table 5-1). The steady-state levels of GH receptor mRNAs as reflected by their autoradiographic intensities were highest at day 30 and declined by day 45 in pregnant (Px) gilts (Table 5-1). GH receptor mRNA abundance was nearly constant during days 45-75, but decreased between days 75 and 90 and remained at reduced levels through day 4 postpartum (Figure 5-1 and Table 5-1). Pseudopregnant (Ppx) control gilts had lower levels of GH receptor mRNAs in mammary tissues than did the corresponding Px gilts. The GH receptor mRNA level in estradiol-treated Ppx gilts was 5.5-fold lower than that for the Ppx control group at day 60, but not at day 112 (day 0 = onset of estrus).

Northern Analysis of IGF-I and IGF-II mRNAs

IGF-I mRNAs with lengths of 8, 6, 4.5, and 1 kb were detected by Northern analysis of mammary poly (A^+) RNA preparations (Figure 5-2). The 8 kb RNA was the predominant IGF-I mRNA species detected in these RNA preparations. The level of the 8 kb transcript was nearly constant during days 30-75 of pregnancy except for a slight decline at day 60, declined 2.5-fold by day 90, and subsequently increased to ~70% of that for day 30

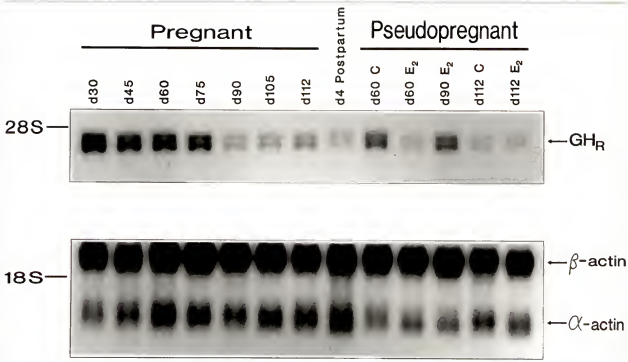

Figure 5-1. Northern analysis of GH receptor and ß-actin mRNAs. Five micrograms of poly(A$^+$) RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled EcoRI-XhoI fragments (1.1 and 1.8 kb) of pGH receptor cDNA clone pMON 2268. Each RNA preparation was extracted from pooled mammary tissues from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy. The filter was rehybridized with ^{32}P-labeled chicken ß-actin cDNA fragment (bottom). Top and bottom panels are positioned to scale.

Table 5-1. Relative Abundance of GH Receptor and ß-Actin mRNAs in Mammary Tissues[a]

		Growth Hormone Receptor	
	ß-Actin	Uncorrected	Normalized to ß-actin
Pregnant (Px)			
day 30	100.0	100.0	100.0
day 45	86.6	63.1	72.9
day 60	122.1	85.0	69.9
day 75	132.6	76.2	57.5
day 90	114.9	26.2	23.5
day 105	120.7	27.3	22.6
day 112	114.1	39.0	35.0
day 4 (postpartum)	134.7	23.3	17.3
Pseudopregnant (Ppx)			
day 60 C	131.0	69.6	53.1
day 60 E_2	123.0	11.8	9.6
day 90 E_2	136.1	64.0	47.0
day 112 C	120.8	26.4	21.9
day 112 E_2	119.8	23.0	19.2

[a]Relative abundance was determined by autoradiographic intensity of each mRNA band in Figure 5-1. For each autoradiogram, the autoradiographic intensity of the band for day 30 Px mammary tissue was arbitrarily assigned as 100%.

by day 112 (Table 5-3). The mammary levels of IGF-I mRNAs in Ppx control gilts were lower than for the corresponding Px gilts and declined between days 60 and 112 (Figure 5-2 and Table 5-3). Estradiol administration reduced mammary IGF-I mRNA level at day 60, but increased it by day 112. IGF-I mRNA levels in mammary tissues were comparable to those in neonatal pig liver (Figure 5-2).

Multiple species of IGF-II mRNAs with sizes of 6.4, 4.4, 2.4, 1.6, and 1.3 kb were detected by Northern analysis of mammary poly(A^+) RNA (Figure 5-3). The IGF-II mRNAs were also detected in total RNA preparations from mammary tissues at days 12 and 30 of Px, the levels of which were comparable (Figure 5-3 and Table 5-3). The relative abundance within an RNA preparation of each IGF-II mRNA was constant with the 2.4 kb mRNA being most abundant, followed by the 4.4 kb mRNA species. The levels of 2.4 kb IGF-II mRNA in Px gilts were highest at day 30 and declined in a step-wise manner at days 45 and 75 (Table 5-3). As in previously described Northern analyses, the mammary IGF-II mRNA levels for Ppx control gilts were lower than those for Px gilts at both days 60 and 112. Estradiol administration decreased IGF-II mRNA abundance at day 60, but had no effect at day 112.

Concentrations of IGF-I and IGF-II in Mammary Tissues and Sera

To assess the temporal change in tissue IGF concentration, mammary tissues were extracted with acetic acid followed by acid-ethanol and IGF RIAs were performed. IGF measurements were corrected for the contributions from serum (1.7 ± 0.1%, v/w of tissue). This represented 16.2 ± 3.3% of IGF-I and 7.7 ± 0.8% of IGF-II in days 12-75 mammary tissues and 3.2 ± 0.6% of IGF-I and 5.4 ± 0.6% of IGF-II in mammary

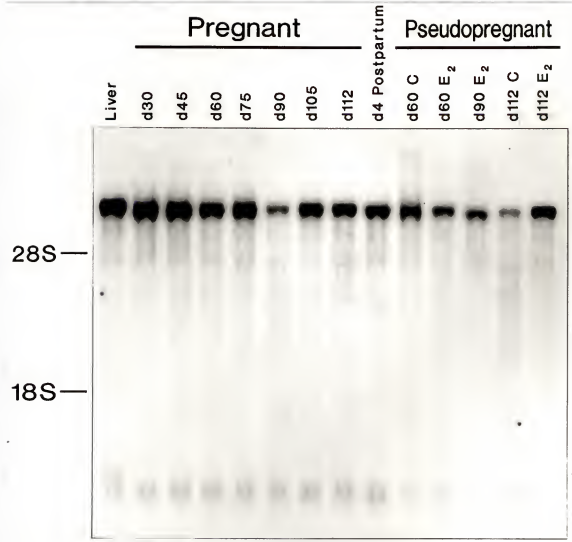

Figure 5-2. Northern analysis of IGF-I mRNAs. Five micrograms of poly(A$^+$) RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled pIGF-I cDNA fragment. Each RNA preparation was extracted from pooled mammary tissues or livers from three pregnant (Px) gilts or 21-day-old piglets or from two of each group of pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg estradiol (E_2) valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy.

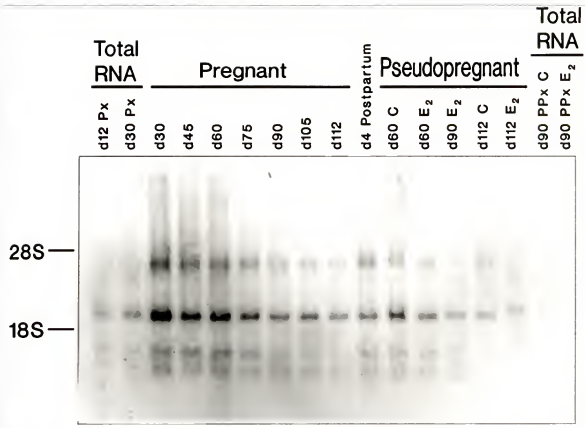

Figure 5-3. Northern hybridization of IGF-II mRNAs. Five micrograms of poly(A^+) or 40 μg of total RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled, 780 bp PstI fragment of rIGF-II cDNA. Each RNA preparation was extracted from pooled mammary tissues from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy.

Table 5-2. Concentrations of IGF-I and IGF-II in Mammary Tissues and Sera

Status	IGF-I Tissue (ng/gm)	IGF-I Serum (ng/ml)	IGF-II Tissue (ng/gm)	IGF-II Serum (ng/ml)
Pregnant[a]				
day 12	7.0±2.1	ND[b]	79.6±12.6	ND
30	3.8±0.7	86.3±4.9	64.9±15.7	306.7±24.6
45	8.2±0.4	84.4±7.4	74.2±7.3	317.4±15.8
60	7.6±1.5	71.6±9.9	70.5±4.8	304.5±14.3
75	8.7±0.5	64.7±11.9	65.9±8.5	284.0±14.8
90	20.2±3.8	41.8±5.1	99.9±16.3	253.4±15.9
105	48.5±11.0	43.6±5.4	403.4±300.0	249.4±14.1
112	56.8±10.7	37.5±9.1	226.6±60.6	232.5±19.9
4 (postpart.)	84.6[c]	62.8±5.3	402.3[c]	276.0±0.7
Pseudopregnant[d]				
day 60 C	30.4±7.7	83.3±19.8	294.5±34.9	336.7±26.3
60 E_2	37.6±8.7	86.0±15.6	99.6±2.5	268.4±23.3
day 90 C	ND	97.4±13.1	ND	351.0±16.6
90 E_2	61.4[e]	94.1±20.4	247.1[e]	324.5±6.1
day 112 C	155.0±141.4	114.6±14.4	355.9±244.9	345.5±48.1
112 E_2	41.8±13.7	96.3±11.1	213.6±20.0	264.7±31.1

[a]Data are means ± SEM of three observations. Mean IGF-I concentrations in mammary tissues and sera and mean IGF-II concentrations in sera differed among days at $P < 0.01$ and $P < 0.05$, respectively.
[b]ND, not determined.
[c]Single observation.
[d]Received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy. Data are means ± SEM of two observations. Effect of day, treatment or day x treatment was not significant ($P > 0.05$).
[e]Represents the concentration for a pooled tissue from two animals.

tissues of late Px and Ppx gilts. IGF-I concentrations in mammary tissues of Px gilts were relatively constant up to day 75, increased by 2.3-fold by day 90, and further increased by 4-fold during the rest of Px and postpartum (Table 5-2); this trend was different from the temporal pattern of mammary IGF-I mRNA expression. Mean mammary IGF-I concentration differed significantly among days ($P < 0.01$). Mammary tissue IGF-II concentrations were approximately 10-fold greater than IGF-I concentrations. They increased 1.5-fold between days 75 and 90 of Px and further increased 4-fold between day 90 of Px and day 4 postpartum (Table 5-2). Although mean mammary IGF-II concentrations were not different among days ($P = 0.23$), the mean IGF-II concentration for days 90-112 of Px and day 4 postpartum was greater than that for earlier days of Px ($P < 0.05$). Ppx gilts, in general, had greater mammary concentrations of both IGFs than did Px gilts at corresponding days after onset of estrus. However, neither a temporal variation nor an effect of estradiol on mammary tissue IGF concentrations was detected.

Serum concentrations of IGF-I in Px gilts declined slightly prior to day 75, declined markedly between days 75 and 90 and thereafter remained constant. Mean serum IGF-I concentrations differed among days ($P < 0.01$). Serum IGF-II levels also declined during Px ($P < 0.05$), although the relative decrease was less than that for IGF-I. Circulating levels of both IGFs in Ppx gilts were similar to those of early Px gilts and did not change either with duration of Ppx or in response to estradiol administration.

Type I and Type II IGF Receptors

Type I IGF receptor mRNAs with lengths of 11, 7, and 6 kb were identified after Northern blot hybridization of mammary poly(A^+) RNAs with a human Type I IGF receptor cDNA probe (Figure 5-4A). The 11 kb mRNA was the most abundant species, but the relative abundance of each size class of mRNA was variable among the individual RNA preparations. Temporal variations in abundance of Type I IGF receptor mRNAs for Px and Ppx gilts were similar to those for IGF-I mRNAs, except that levels of the Type I receptor mRNAs were highest at day 12 of Px and increased transiently at day 75 of Px (Table 5-3). Affinity-crosslinking of mammary microsomal membrane proteins with radiolabeled IGF-I identified a 135 kDa band representing the cross-linked complex of IGF-I and the α-subunit of the Type I IGF receptor (Figure 5-4B). In addition to the 135 kDa α-subunit, a 170 kDa protein band was invariably detected by affinity-crosslinking. Radiolabeled IGF-I was almost completely displaced from the receptors by addition of approximately 100-fold unlabeled IGF-I, was mostly displaced by unlabeled IGF-II, but was not displaced by insulin at this dose (Figure 5-4B).

A single Type II IGF receptor mRNA of 9 kb length was detected by Northern blot analysis (Figure 5-5A). The relative abundance of this mRNA in mammary tissue was similar to that for Type I IGF receptor mRNAs (Figure 5-4A). Western ligand blotting of mammary microsomal membranes using radiolabeled IGF-II identified a 250 kDa Type II IGF receptor protein (Figure 5-5B). Membrane Type II IGF receptor content was highest at day 30 of Px, otherwise, temporal variations in this receptor protein followed the changes in Type II receptor mRNA levels. Affinity-

A.

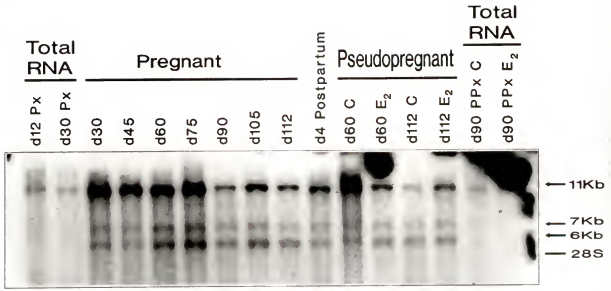

Figure 5-4. Northern blot-hybridization and affinity-crosslinking of mammary Type I IGF receptor mRNAs and proteins. A. Five micrograms of poly(A^+) or 40 μg of total RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled EcoRI fragment (730 bp) of human Type I IGF receptor cDNA. Each RNA preparation was extracted from pooled mammary tissues from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy. B. One milligram of membrane proteins, prepared from pooled mammary tissues, was incubated with 1 X 10^6 cpm [^{125}I]IGF-I (1 ml) overnight at 4 C with or without 0.2 μg unlabeled IGF or insulin, cross-linked with DSS (0.1 mM) for 30 min at 4 C, electrophoresed in a 7.5% SDS-polyacrylamide gel under reducing conditions (5% 2-mercaptoethanol), and subjected to autoradiography.

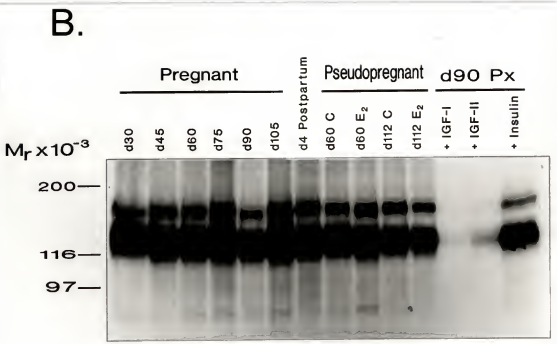

Figure 5-4--continued

Table 5-3. Relative Abundance of IGF-I and IGF-II and Type I IGF Receptor mRNAs in Mammary Tissues[a]

Status	IGF-I	IGF-II	Type I Receptor
Pregnant (Px)			
day 12 (tot. RNA)	ND[b]	ND	27.9
day 30 (tot. RNA)	ND	ND	15.4
day 30	100.0	100.0	100.0
day 45	98.0	51.4	76.2
day 60	74.2	60.1	75.7
day 75	92.9	30.3	131.3
day 90	37.1	23.1	52.1
day 105	78.4	30.2	92.5
day 112	66.5	29.6	62.2
day 4 (postpartum)	72.7	37.8	58.4
Pseudopregnant (Ppx)			
day 60 C	63.6	52.6	63.3
day 60 E_2	48.9	29.7	52.8
day 90 E_2	50.9	ND	ND
day 112 C	27.9	23.2	48.1
day 112 E_2	59.1	25.0	60.2

[a]Relative abundance was determined from autoradiographic intensity of the most intense IGF-I (Figure 5-2) and IGF-II (Figure 5-3) mRNA bands and the sum of all mRNA bands for Type I IGF receptor Northern (Figure 5-4). In each autoradiogram, the intensity for day 30 Px mammary tissue was arbitrarily assigned as 100%.
[b]ND, not determined.

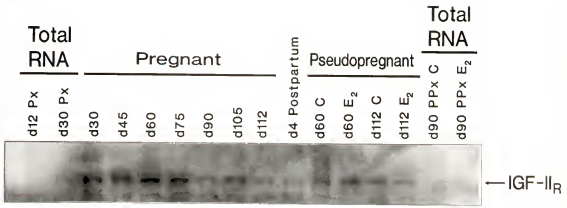

Figure 5-5. Northern analysis, Western ligand blot-analysis and affinity-crosslinking of mammary Type II IGF receptor mRNAs and proteins. A. Five micrograms of poly(A^+) RNA or 40 μg of total RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled, 3kb EcoRI fragment of rat Type II IGF receptor cDNA. Each RNA preparation was extracted from pooled mammary tissue from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy. B. Solubilized membranes (150 μg protein) prepared from pooled tissues were electrophoresed in a 7.5% SDS-polyacrylamide gel under nonreducing conditions and proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked for 24 h at 4 C in 1% nonfat dry milk, incubated with [^{125}I]IGF-II (80,000 cpm/ml) for 3 h at room temperature, washed and subjected to autoradiography. C. Membrane proteins (800 μg) prepared from pooled mammary tissues were incubated with 6 X 10^5 cpm [^{125}I]IGF-II (1 ml) overnight at 4 C with or without 0.5 μg unlabeled IGF or insulin, cross-linked with DSS (0.1 mM), electrophoresed in a 7% SDS-polyacrylamide gel under reducing conditions (5% 2-mercaptoethanol), and subjected to autoradiography.

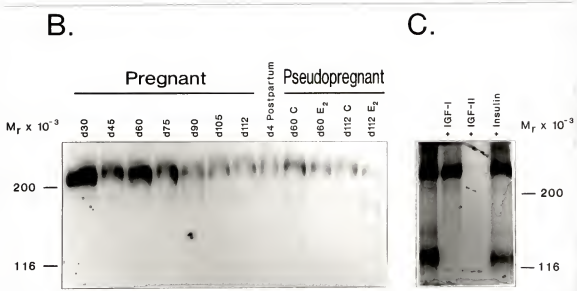

Figure 5-5--continued

crosslinking of microsomal membrane proteins with radiolabeled IGF-II identified the 250 kDa Type II IGF receptor as well as the 135 kDa α-subunit of the Type I IGF receptor (Figure 5-5C). Labeling of both bands was abolished by addition of excess unlabeled IGF-II, whereas only the labeled 135 kDa band was abolished by excess unlabeled IGF-I. Insulin at the same dose diminished the intensity of the 135 kDa band, but had no effect on the intensity of the 250 kDa Type II IGF receptor band.

Expression of IGFBP-2 and IGFBP-3 in Mammary Glands and Sera

Unlike the temporal variations observed for the other mRNAs, the mammary tissue abundance of IGFBP-2 mRNA (1.5 kb) in Px gilts exhibited a biphasic pattern (Figure 5-6A), i.e., elevated at days 60 and 75 followed by a decline at days 90 and 105 and a second rise by day 112. The IGFBP-2 mRNA levels in mammary tissues of Ppx gilts were generally lower than those in Px gilts. However, Ppx gilts had an elevated level of this mRNA at day 112 which was unaffected by estradiol treatment. The temporal pattern for mammary expressed IGFBP-3 mRNA was unique among the IGF family of mRNAs examined (Figure 5-6B). The level of this ~3 kb mRNA declined abruptly after day 30 of Px and was below detection by day 105. Moreover, this mRNA was undetectable in mammary glands of Ppx gilts.

Serum IGFBPs were identified by Western ligand blotting using radiolabeled IGF-II (Figure 5-6C). In contrast to the abrupt decline in mammary IGFBP-3 mRNA levels after day 30 of Px, the level of IGFBP-3 (proteins with molecular mass of 43 and 40 kDa) in serum began to decline only after day 60 of Px. Serum IGFBP-2 (34 kDa IGFBP) levels, which did not exhibit any obvious temporal trend, were also not correlated with mammary IGFBP-2 mRNA levels. Serum levels of IGFBP-2 and IGFBP-3 did not

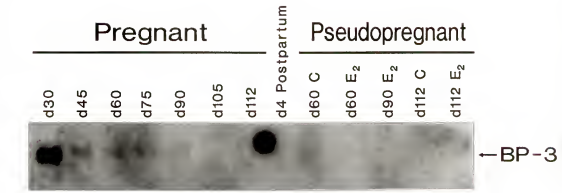

Figure 5-6. Northern analysis of mammary IGFBP-2 and IGFBP-3 mRNAs and ligand blot analysis of serum IGFBPs. A,B. Five micrograms of poly(A$^+$) or 40 μg of total RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled HindIII fragment (1.2 kb) of an IGFBP-2 cDNA subclone (A) or with a ^{32}P-labeled EcoRI insert (2.6 kb) from a hIGFBP-3 cDNA clone (B). Each RNA preparation was extracted from pooled mammary tissue from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through the day preceding the mammary biopsy. C. Three microliters of serum were electrophoresed in a 12.5% SDS-polyacrylamide gel, proteins were electrotransferred to a nitrocellulose membrane, incubated with [^{125}I]IGF-II (120,000 cpm/ml) overnight at 4 C and the membrane was washed and subjected to autoradiography.

C.

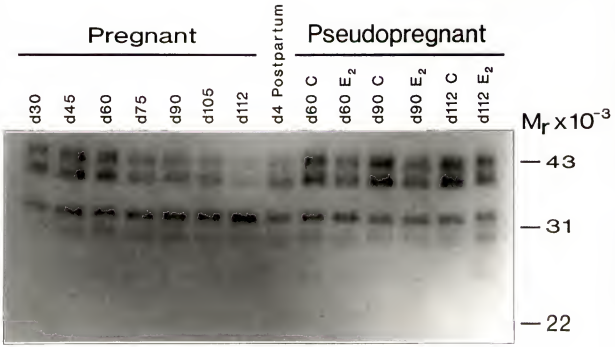

Figure 5-6--continued

change with day of Ppx. However, estradiol administration led to a reduction in serum IGFBP-3 levels, but had no effect on serum IGFBP-2 levels.

Lactogenesis

To temporally relate onset of lactogenesis to tissue expression of IGFs and their receptors and IGF content, Northern analysis was performed using rodent ß-casein cDNA probes. A ß-casein mRNA of 1.35 kb was detected in mammary glands of late Px (> day 90) and Ppx gilts (days 60-112) (Figure 5-7). Tissue levels of this mRNA increased to term and then declined after parturition. In Ppx gilts, ß-casein mRNA was detected in every RNA preparation and estradiol treatment increased the levels of this mRNA.

DISCUSSION

The present study revealed that steady-state levels of mRNAs encoding IGFs-I and -II, Types I and II IGF receptors and GH receptors were higher during mammogenesis than during lactogenesis, with a clear demarcation between the two stages at day 90 of Px. This indicates that expression of components of the mammary IGF system is down-regulated with onset of lactogenesis and differentiation of mammary epithelial cells. Consistent with this view, the premature lactogenesis (day 60), as well as the reduced mammogenesis in Ppx gilts compared to Px gilts (Kensinger et al., 1986), was associated with reduced levels of these mRNAs. These results suggest, but do not prove, that the IGFs have a role in mediation of mammogenesis in the pig. The timing of elevated levels of Type I IGF receptor mRNAs at days 12 and 75 when DNA synthesis in mammary tissue is active (Kensinger et al., 1982) also supports this suggestion.

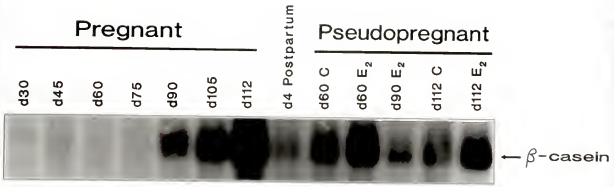

Figure 5-7. Northern hybridization analysis of ß-casein mRNA. Five micrograms of poly(A^+) RNA were electrophoresed in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled, PstI-digested plasmids containing a 800 bp mouse ß-casein cDNA fragment (pCMß13) and a 600 bp rat ß-casein cDNA fragment (6ßc3'). Each RNA sample was extracted from a pool of mammary tissues from three pregnant (Px) or two pseudopregnant (Ppx) gilts. Ppx gilts received a daily injection of 5 mg E_2 valerate or vehicle (C) from day 45 through to the day preceding the mammary biopsy.

Temporally regulated expression of IGFs and IGF receptors in porcine mammary tissues was similar to that reported for rat mammary glands. In rats, levels of membrane Type I IGF receptors peaked at day 5 of Px and then declined during later pregnancy and lactation (Lavandero et al., 1990) and mammary IGF-I mRNA levels were greater during the first half of pregnancy than during the second half (Marcotty and Maghuin-Rogister, 1991), which also suggested a role for IGF-I in mammogenesis.

Results of IGF receptor analyses indicated that IGF-I binds to only the Type I IGF receptor, whereas IGF-II binds to both types of IGF receptors. The order of affinities of the Type I IGF receptors for these ligands (IGF-I > IGF-II > insulin) and the selective affinity of the Type II receptor for IGF-II were consistent with previously reported results for bovine mammary tissue (Hadsell et al., 1990). The minor protein band with an estimated molecular mass of 170 kDa observed in affinity-crosslinking of membrane proteins with [^{125}I]IGF-I may have represented a glycosylation variant of the α-subunit of the Type I IGF receptor.

The growth-promoting action of both IGFs are mediated via Type I receptors in most cells and tissues (Van Wyk et al., 1985; Conover et al., 1986), suggesting that mammogenic action of both IGFs is likely to be mediated via this receptor type. However, a potential role for Type II IGF receptors in mediating this response cannot be completely ruled out, as IGF-II reportedly stimulates in vitro growth of human breast cancer cells via both types of IGF receptors depending on the concentration of this peptide in the culture medium (Mathieu et al., 1990). If the Type II IGF receptor mediates the mitogenic response in porcine mammary tissue,

IGF-II may have a preferential role in early mammogenesis when the levels of both IGF-II and Type II receptor mRNAs are high.

IGFBPs are IGF carriers and potential modulators of IGF action at the cell surface (Baxter and Martin, 1989a; Ooi, 1990). Although physiological roles for individual IGFBPs are presently a subject of active investigation, IGFBP-3 may serve primarily as an endocrine carrier of IGF, whereas IGFBPs-1 and -2, by virtue of their RGD sequences which may mediate binding to the cell surface integrin receptors (Hynes, 1987), may be important local modulators of IGF bioactivity (Ooi et al., 1990). In this regard, the increased mammary expression of IGFBP-2 mRNA at days 60 and 75 of Px, along with the decrease in IGFBP-3 mRNA expression on these same days, may serve to increase the local availability of IGFs. The increased levels of IGFBP-2 mRNA during the immediate peri-parturient period in Px gilts and at day 112 in Ppx gilts were associated with increased mammary tissue concentrations of IGFs, suggesting that locally synthesized IGFBP-2 may serve as major vehicle for sequestration of IGFs in mammary tissues or in mammary secretions (Simmen et al., 1988b).

The present study did not define the specific mammary cell types which expressed IGF mRNAs. Results from other studies revealed that the IGFs are expressed predominantly or exclusively in stromal cells in human and bovine mammary tissues (Glimm et al., 1988; Yee et al., 1988, 1989; Campbell et al., 1991), whereas sheep mammary epithelial cells synthesize IGF-I (Wheatley et al., 1989). Types I and II IGF receptors were detected in mammary tissues and breast cancer cells (Yee et al., 1988; Hadsell et al., 1990; Mathieu et al., 1990) and IGFBPs are present in conditioned culture medium of mammary epithelial cells (Baxter, 1983; Campbell et al.,

1991). Taken together, autocrine and paracrine actions of IGFs and IGFBPs on mammary epithelial cells are likely.

Estrogen appears to be a lactogenic hormone in the pig as indicated by the enhanced ß-casein mRNA expression in estradiol-treated Ppx gilts. The timing of onset of lactogenesis in Px gilts (day 90) was also consistent with the plasma estrogen profile, since estrogen levels increase 4.5-fold between days 75 and 90 of Px (DeHoff et al., 1986). In this regard, the decline in levels of mRNAs for IGFs, IGF receptors and GH receptors between days 75 and 90 in Px gilts was similar to that observed at day 60 of Ppx gilts after 15 days of estradiol administration, although levels of IGF-I and Type I receptor mRNAs tended to increase after long-term estradiol administration. Since estrogen is synthesized and released primarily from placenta in increasing amounts during late pregnancy in this species (Knight et al., 1977), estrogen may be a conceptus signal that initiates cessation of mammogenesis, differentiation of mammary epithelial cells, and onset of lactogenesis. However, since mammary DNA concentrations increase 4-fold between days 75 and 90 of Px (Kensinger et al., 1982), it also seems plausible that estrogen accelerates both mammogenesis and lactogenesis during this period. In line with this possibility, stradiol-treated Ppx gilts had apparently greater mammary development than Ppx control gilts by day 90, although mammary growth index was not measured in this study. In this connection, the effects of estrogen on mammary development may be, in part, mediated via prolactin, since prolactin receptor concentrations in mammary tissues are temporally related to changes in circulating estrogen concentrations (M. H. DeHoff, R. J. Collier, F. W. Bazer, and H. N. Becker, unpublished results).

The coincidental increase in mammary gland IGF concentrations and ß-casein mRNA accumulation suggests that IGF may also function to initiate lactogenesis, as surmised from the lactogenic activity of IGF-I observed both in vivo and in vitro (Baumrucker, 1986; Prosser et al., 1987, 1990). The high IGF values for lactogenic stage mammary glands are likely to have reflected the amounts of sequestered IGFs in precolostral secretions, since precolostral and/or colostral secretions have IGF concentrations that are far greater than those for maternal sera in the pig and cow (Malven et al., 1987; Simmen et al., 1988b). It remains as a possibility, however, that part of the high IGF values may also be related to the increased number of epithelial cells and the increased development of membrane that occcur during late pregnancy (Kensinger et al., 1982, 1986b).

The discrepancy between the temporal patterns of mammary expressed IGF and IGFBP mRNAs and IGF proteins and serum levels of these proteins indicates that expression of components of the mammary IGF system is regulated differently from that of other tissues. However, a role for systemic IGFs in mammary gland development is not excluded. The mammary gland is a known target organ of circulating IGF-I in sheep (Hodgkinson et al., 1991). It is unclear, however, if the decline in serum IGF levels during late Px was associated with sequestration of IGFs by mammary glands. Serum IGF-I levels also decline during late pregnancy in rats (Gargosky et al., 1990b), but increase in pregnant women during the last trimester (Gargosky et al., 1990a). Gargosky et al. (1990b) postulated that the decline in serum IGF-I level during rat pregnancy is related to the need for catabolism in the dam to support growth of a greater mass of

conceptuses per unit body weight when compared to pregnant women (Gargosky et al., 1990a). However, this does not seem to be the case for the pig, since pregnant gilts are not in a state of negative energy balance as evidenced by increases in body weight throughout pregnancy (Clawson et al., 1963).

Northern analysis identified a 4.6 kb mRNA doublet with sequence homology to porcine GH receptor cDNA. A smaller-sized mRNA, encoding a truncated GH receptor (serum GH-binding protein) in the rat (Baumbach et al., 1989), was not identified in the present study. Since the probe used contain all the coding sequence, porcine serum GH-binding proteins (Daughaday et al., 1987b) may be proteolytically generated from entire GH receptors translated from the GH receptor mRNAs detected in this study. A potential role for GH receptors in the mediation of IGF expression in mammary glands was less than clear. The temporal pattern of mammary GH receptor mRNA expression somewhat resembled that for IGF-I mRNAs, but a correlation between these two variables was not as apparent as that for IGF-I and Type I receptor mRNAs. Attempts to identify GH receptor proteins in mammary microsomal membranes by radioreceptor assay or by affinity-crosslinking were unsuccessful (data not shown). Although GH receptor mRNAs were previously detected in mammary tissues (Hauser et al., 1990; Jammes et al., 1991), identification of mammary GH receptor proteins has been elusive (Gertler et al., 1984; Ackers, 1985). Thus, it remains to be demonstrated whether the porcine GH receptor mRNAs are translated into functional receptor proteins in this tissue.

CHAPTER 6

SUMMARY AND CONCLUSIONS

In the first experiment, the ontogeny of serum IGFs-I and -II and IGFBPs during fetal and postnatal development was studied. In so doing, an IGF separation procedure using acidification and C_{18} Sep-Pak chromatography was validated for porcine serum by demonstrating the complete removal of IGFBPs and parallelism in both IGF RIAs. Results of the RIAs for porcine serum revealed that IGF-I levels are low during fetal life and increase during postnatal development, whereas IGF-II levels are high during the fetal period and also increase postnatally. These results support the view that IGF-II is a fetal and postnatal growth mediator in the pig, whereas IGF-I may be primarily a postnatal growth factor. However, this does not exclude a potential role for IGF-I in fetal growth, since tissue expression of IGF-I was not measured in this experiment. Overall, the ontogeny of both IGFs in porcine serum was similar to that reported for human serum (Zapf et al., 1981).

Serum IGFBPs with apparent molecular mass of 43 kDa, 39 kDa, 34 kDa, 31 kDa, and 26 kDa were identified by the Western ligand blotting. The 43 and 39 kDa IGFBPs were identified as glycosylation variants of IGFBP-3 by deglycosylation, immunoprecipitation and ligand blotting and the 34 kDa IGFBP was shown to be immunologically related to IGFBP-2. The 31 kDa IGFBP band was resolved as a multiplet in ligand blotting. This band thus appears to represent a mixture of IGFBPs-1, -4 and -5 (Shimonaka et al.,

1989; Zapf et al., 1990; Kiefer et al., 1991a; Shimasaki et al., 1991b; McCusker et al., 1991). The pIGFBP-3 immunoprecipitate of serum also exhibited a band at the 31 kDa region in addition to the 43 and 39 kDa bands, indicating that the 31 kDa IGFBP band also includes a truncated form of IGFBP-3 (Walton et al., 1989). By reference to the current literature, the 26 kDa IGFBP appears to be a deglycosylated form of IGFBP-4 (Shimonaka et al., 1989; Kiefer et al., 1991a). Results from the Western ligand blotting indicated that the major IGF carrier in fetal pig serum is the 34 kDa IGFBP which is mostly replaced by the ~40 kDa IGFBPs concomitant with increasing IGF-I concentrations in postnatal serum, whereas the 31 kDa and 26 kDa IGFBPs represent only a minor portion of the total serum IGF-binding activity at all developmental stages. Since it is known that GH negatively regulates the secretion of IGFBP-2 (Coleman and Etherton, 1991; McCusker et al., 1991) and that IGF-I stimulates the secretion of IGFBP-3 both in vivo and in vitro (Zapf et al., 1989; Hill et al., 1989), the peri-natal transition in distribution of serum IGFBPs appears to reflect the maturation of the somatotropic axis. Overall, the ontogeny of IGFBPs in porcine serum was similar to that for the human and rat (Hardouin et al., 1987; Yang et al., 1989).

In the second experiment, expression in tissues of IGFs and IGF receptor proteins and mRNAs encoding IGFs, IGFBPs, IGF and GH receptors was studied along with the ontogeny of serum IGFs and IGFBPs in order to relate these events and to also assess IGF dynamics among tissues. Results on the ontogeny of serum IGFs and IGFBPs were consistent with those from the first experiment in terms of temporal patterns of changes. The lower serum IGF-I concentrations for days 112 fetal and 42 postnatal

pigs compared with corresponding values from the first experiment appear to have resulted from variation among animals.

A surprising finding from this study was that the levels of mRNAs encoding IGF-I in porcine liver were not higher than those for other internal organs and muscle and did not increase during postnatal development. This markedly contrasts with the predominance of IGF-I mRNA expression in rat liver (Lund et al., 1986) and the 100-fold postnatal increase in hepatic IGF-I mRNA levels in rats (Adamo et al., 1989). The postnatal decline in tissue levels of IGF-II mRNAs contrasted with the postnatal increase in serum IGF-II levels. Levels of IGF-II mRNAs in rat tissues also decline postnatally (Lund et al., 1986), but in this species, serum IGF-II levels also decline rapidly after birth (Moses et al., 1980).

Northern, Western and affinity-crosslinking analyses of the IGF receptors revealed that tissue expression of these receptors was reduced postnatally. In particular, the perinatal decline in tissue levels of Type II receptor proteins was pronounced. The persistence of high levels of the Type I receptor activity in the lung may be related to a previously identified developmental increase in receptor affinity (D'Ercole et al., 1976).

Simultaneous analyses of expression of the two IGFs and their receptors allowed an assessment of the potential dynamics of IGF among tissues. Fetal liver is presumed to be a major IGF-II endocrine organ with its high levels of IGF-II mRNAs, whereas fetal lung, exhibiting low levels of IGF-II mRNAs and high levels of Type II receptors, is a potential target of circulating IGF-II. In this connection, relatively high IGF-II concentrations in fetal lungs were reflective of high receptor

levels, suggesting receptor-mediated uptake of systemic IGF-II. Also indicated from the multiple analyses was that skeletal muscle is a potential source of plasma IGF-I in postnatal pigs, since the muscle of postnatal pigs exhibited a relatively high level of IGF-I mRNAs and a low level of Type I receptors.

Results of Northern analyses for IGFBPs-2 and -3 did not indicate that they are produced primarily in the liver as for the rat (Scott et al., 1985a; Baxter and Martin, 1989a). Although liver had the highest levels of IGFBP-2 mRNA which exhibited a temporal trend similar to that for serum IGFBP-2 levels during the latter half of fetal life, the rapid postnatal decline in hepatic IGFBP-2 mRNA levels contrasted with the gradual decline in serum IGFBP-2 levels.

Collectively, the higher levels of IGF receptors and IGF proteins in fetal tissues, albeit somewhat inconsistent in the latter, suggest that both IGFs likely have more important roles in growth of fetuses than of postnatal pigs. The tendency for an inverse relationship between plasma IGF levels and overall tissue levels of IGF receptors indicates that IGFs are likely to be primarily autocrine/paracrine-acting peptides and hence, plasma IGFs may represent mainly a pool of reserve IGFs after local utilization. However, tissue pools of IGFs are likely derived from both local and systemic sources. In this connection, the present results do not support the concept that liver is the major endocrine source of IGFs and IGFBPs in the pig as has been suggested for the rat (Schwander et al., 1983; Scott et al., 1985a). Since levels of mRNAs encoding IGFs-I and -II did not increase during postnatal development in any of the tissues examined, the postnatal increases in serum levels of IGFs in the pig are

likely due to decreased clearance rates of these proteins rather than to their increased hepatic secretion rates. It is hypothesized that the postnatal increases in serum IGF levels in this species are due to the postnatal transition of IGFBP-2 to IGFBP-3 in plasma and the reduced expression of IGF receptors in multiple tissues. Plasma levels of IGFs may be determined by equilibria among rates of IGF synthesis and release, transcapillary movement, and tissue uptake which appears to be regulated in a coordinated way.

In the last part of the study, expression of components of the IGF system in mammary glands and sera was measured during pregnancy (Px) and pseudopregnancy (Ppx) to gain insights into roles for IGFs and estrogen in the pregnancy-associated development of mammary glands. It was established from previous studies that by day 90 of Px, mammogenesis is complete and lactogenesis has been initiated in the pig (Kensinger et al., 1982) and that the presence of conceptuses has a stimulatory effect on mammary growth (Kensinger et al., 1986). From this study, following conclusions are presented. First, mammary levels of mRNAs encoding IGFs-I and -II, Types I and II IGF receptors and GH receptors were higher during mammogenic (days 12-90) than during lactogenic (day 90-term) phases, with the minimum levels consistently observed at day 90 of Px. Thus, the cessation of mammogenesis and initiation of mammary epithelial cell differentiation (lactogenesis) were characterized by the reduced tissue expression of components of the mammary IGF system.

Second, relative to Px gilts, Ppx gilts had reduced mammary expression of mRNAs encoding the IGF-related proteins, which was temporally associated with premature (day 60) synthesis of β-casein mRNA.

This suggested that reduced mammogenesis in Ppx gilts is secondary to premature differentiation of mammary epithelial cells. It thus appears plausible that an unknown factor(s) of conceptus origin allows for maximal mammary growth by delaying differentiation of mammary epithelial cells. Collectively, the present results support a role for locally produced IGFs in the pregnancy-associated growth of mammary glands.

Third, estrogen appears to be a lactogenic signal in the pig as indicated by the increased β-casein mRNA levels following estradiol administration in Ppx gilts. The coincidental synthesis of β-casein mRNA (day 90) and rise in plasma levels of estrogen (DeHoff et al., 1986) in Px gilts are also consistent with this interpretation. The decline in levels of mRNAs encoding IGFs, IGF receptors and GH receptors in response to 15 days exposure to high levels of estrogen (days 75-90 of Px and 45-60 of Ppx, respectively) suggests that estrogen may not only initiate the differentiation of mammary epithelial cells, but also down-regulate mammary tissue expression of these mRNAs. However, since lactogenesis was initiated in Ppx gilts without secondary estradiol administration, conceptuses may also secrete a factor(s) that inhibits estrogen's lactogenic action prior to the appropriate time for initiation of lactogenesis. In any event, differentiation of mammary epithelial cells is likely to be regulated through complicated interactions of multiple factors of both conceptus and maternal origins in the pig.

Fourth, lactogenesis was characterized by increased mammary concentrations of both IGFs. The source and potential role for these sequestered IGFs, however, remain speculative. Finally, differences in the temporal pattern of expression between serum IGFs and IGFBPs and

mammary mRNAs encoding these proteins during Px and Ppx indicate that expression of components of the mammary IGF system is regulated in a unique fashion. However, it remains unclear why serum IGF levels declined during Px, but not during Ppx and what roles the systemic IGFs may have in pregnancy-associated mammary growth and development and maternal metabolism.

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BIOGRAPHICAL SKETCH

Chul-Young Lee was born in South Korea as the seventh child of the Lee family. He received his BS degree in animal science in 1981 from Seoul National University. He began his graduate studies in August, 1985, at Clemson University under the guidance of Dr. Donald M. Henricks and received the MS degree in nutrition in August, 1988. After nine months at the Ohio State University as a Ph.D. student of Dr. Frank A. Simmen, he moved with Dr. Simmen to the University of Florida in June, 1989, to continue his Ph.D. studies in animal science. He has been married to Sook-Ran for eight years and has three children.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Frank A. Simmen, Chair
Associate Professor of Animal Science

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Fuller W. Bazer
Graduate Research Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul W. Chun
Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

H. Herbert Head
Professor of Dairy Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rosalia C. M. Simmen
Associate Professor of Animal Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1992

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School